THE ROLE OF ANKYRIN REPEAT AND SOCS BOX CONTAINING PROTEIN 4 (ASB4)
IN TROPHOBLAST DIFFERENTIATION AND PSEUDOVASCULOGENESIS

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ABSTRACT

WH Davin Townley-Tilson: The Role Of Ankyrin Repeat And SOCS Box Containing Protein 4 (ASB4) In Trophoblast Differentiation And Pseudovasculogenesis (Under the direction of Cam Patterson)

Vascularization of the placenta is a critical developmental process that ensures fetal viability. The vascular health of the placenta affects both maternal and fetal well being; however, relatively little is known about the early stages of placental vascular development. The ubiquitin ligase Ankyrin repeat, SOCS box-containing 4 (ASB4) promotes embryonic vascular lineage commitment and is highly expressed early in placental development. The transcriptional regulator Inhibitor of DNA binding 2 (ID2) negatively regulates trophoblast differentiation during development and is a target of many ubiquitin ligases. Due to their contrasting effects during differentiation, we investigated whether ASB4 mediates vascular differentiation through its ligase activity on ID2 in the placenta. Placentas from Asb4−/− mice exhibited myriad vascular differentiation defects, including abnormal overexpression of ID2, and pregnant Asb4−/− mice phenocopied human pre-eclampsia. We determined that ASB4 directly interacted with ID2 in trophoblast cells, leading to ID2’s ubiquitination and subsequent degradation. Further, ASB4 promoted placental cell differentiation and function, and co-expression of a degradation-resistant Id2 mutant abolished these effects. Together, these findings indicate that ASB4 regulates trophoblast cell differentiation into placental vasculature through the degradation of ID2 and that loss of Asb4 in the developing placenta contributes to placental disease.
This work is dedicated to mother, who wasn’t able to see me complete my goal; my father, who instilled the value of education in me; my brother, who has always been smarter than me; and my wife, who makes me better every day.
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<tr>
<td>APC/C</td>
<td>Anaphase promoting complex/cyclosome</td>
</tr>
<tr>
<td>ASB4</td>
<td>Ankyrin repeat and SOCS containing protein 4</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>cKit</td>
<td>Kit oncogene, a/k/a stem cell growth factor receptor</td>
</tr>
<tr>
<td>CTB</td>
<td>Cytotrophoblast</td>
</tr>
<tr>
<td>dbcAMP</td>
<td>N(6),2'-O-dibutyryladenosine 3':5' cyclic monophosphate</td>
</tr>
<tr>
<td>D-box</td>
<td>Destruction box motif</td>
</tr>
<tr>
<td>DR</td>
<td>Degradation-resistant</td>
</tr>
<tr>
<td>DS</td>
<td>Degradation-sensitive</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic days post fertilization</td>
</tr>
<tr>
<td>E1</td>
<td>Ubiquitin activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>Ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Ubiquitin ligase</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoetin</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem (cell)</td>
</tr>
<tr>
<td>FIH</td>
<td>Factor inhibitin HIF</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HELLP</td>
<td>Hemolysis, elevated liver enzymes, low platelet count</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factors</td>
</tr>
<tr>
<td>HLH</td>
<td>Helix-loop-helix</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ID2</td>
<td>Inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine growth restriction</td>
</tr>
<tr>
<td>JAR</td>
<td>Choriocarcinoma cell line</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>KDM1</td>
<td>Lysine (K)-specific demethylase 1</td>
</tr>
<tr>
<td>LL</td>
<td>Lysine-less</td>
</tr>
<tr>
<td>MEK1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma gene</td>
</tr>
<tr>
<td>RING-finger</td>
<td>Really interesting new gene domain that binds zinc cations</td>
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<tr>
<td>ROC1</td>
<td>A RING-finger E3 ligase encoded by Rbx</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor ligand</td>
</tr>
<tr>
<td>SCFR</td>
<td>Mast/stem cell growth factor receptor, a/k/a cKit</td>
</tr>
<tr>
<td>sFlt1</td>
<td>Soluble-Fms-like tyrosine kinase 1</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>TB</td>
<td>Trophoblast</td>
</tr>
<tr>
<td>TBSC</td>
<td>Trophoblast stem cell</td>
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<tr>
<td>TGC</td>
<td>Trophoblast giant cell</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER 1
GENERAL INTRODUCTION

Vasculogenesis, pseudovasculogenesis, and trophoblast differentiation\(^1\)

Blood vessel formation is classically divided into two categories: vasculogenesis and angiogenesis. Vasculogenesis is the formation of new blood vessels from the \textit{de novo} production of endothelial cells. 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 development.

Vasculogenesis is further divided into two categories: extraembryonic, that occurs in the yolk sac (which functions as the source of early blood cell formation in the developmental blood circulatory system) and allantois, (which gives rise to the umbilical vasculature) and embryonic, i.e. restricted to the embryo itself [1]. Extraembryonic blood vessel formation precedes embryonic vasculogenesis and provides communication between the fetal circulation and the yolk sac to facilitate the transfer of nutrients and blood gases to the developing embryo, and ultimately gives rise to the placenta [2]. In mammals, blood islands assembling within the mesodermal layer of the yolk sac are the first occurrence of vasculogenesis. Blood islands are foci of hemangioblasts that differentiate \textit{in situ}, forming a

\footnote{1 A version of this work was previously published as Townley-Tilson WHD, Wu Y, Ferguson JE III, Patterson C. The ubiquitin ligase ASB4 promotes trophoblast differentiation through the degradation of ID2. PLoS ONE 9(2): e89451. doi:10.1371/journal.pone.0089451}
loose inner mass of 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 [2]. The chorion is the outermost layer of extraembryonic mesoderm and trophectoderm, surrounding the embryo and all other components of the conceptus. The chorionic villi, which arise from the chorion, are small projections that intercalate the uterine tissue, maximizing the surface area in contact with maternal blood [3]. Extraembryonic vasculogenesis in Eutherians (placental mammals) also supplies the allantois with primitive vessels in preparation for chorion fusion and umbilical vessel formation, thus initiating the vascular connection between the fetal and maternal placental tissues [4]. This allantois-chorion fusion is what is responsible for the placenta proper, whereby the allantois and umbilical vasculature joins the chorion, which has now intertwined with the maternal vasculature, forming a network of vessels that will supply the fetus with nutrients and oxygen, and carry away waste from the fetus into the maternal blood stream where it can be eliminated [3]. This vascularization of the early placenta is crucial for the health and viability of not only the fetus, but also the mother (Figure 1.1) [5-7].

Embryonic vasculogenesis (sometimes referred to as intraembryonic vasculogenesis) occurs throughout most of the embryonic mesoderm. The endocardium and great vessels are the first embryonic endothelial structures formed during development [2]. Parallel with 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 [8]. 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 and the pro-epicardium make contact with the developing heart tube and quickly spread over the entire heart, giving rise to the capillaries, veins, and arteries of the coronary vasculature, smooth muscle cells and pericytes [9]. Lastly, the capillary plexi of endothelial cells are remodeled into a unidirectional circuit to allow proper blood circulation throughout the embryo, forming the mature vasculature. To accomplish this vascular specification, endothelial cells are specified into an arterial or venous fate; then, as blood flow commences, vascular smooth muscle cells are recruited to the endothelium to provide structural support and elasticity to the now mature vasculature [10].

The junction of embryonic, extraembryonic, and maternal vasculature is the placenta. Though the placenta is the only transient organ in the body, it requires a great deal of specification for its development, especially within the context of the placental vasculature, during its short window of development to ensure fetal and maternal wellness. Placental vasculogenesis is similar compared with that of embryonic and extraembryonic vasculogenesis in that endothelial cells must differentiate from progenitor cells to form the vasculature. However, the initial progenitor cell reservoir is different, and many of the differentiating endothelial cell characteristics are unique to the placenta. These characteristics include different cell adhesion molecules [11] and intracellular markers of differentiation [12].

The development of the placental vasculature involves many coordinated steps, beginning in early gestation. The outer layer of the blastocyst is a population of trophoblast cells, or the trophectoderm, which will form the extraembryonic and placental components of the conceptus unit. During human pregnancy, a population of undifferentiated multipotent placental cells, termed cytotrophoblasts (CTBs), differentiate into villous and extravillous
trophoblasts that form and remodel the placental vasculature [13]. Villous trophoblasts have endothelial cell functions in the chorionic villi and also fuse into syncytiotrophoblasts, which are the epithelial covering of these villi that penetrate the uterus [14]. Extravillous trophoblasts invade and migrate through the junctional zone of the placenta into the maternal decidua, where they replace the endothelial cells that line the spiral arteries in a process called “pseudovasculogenesis” [6]. These differentiating CTBs “switch” integrin expression profiles from one expression pattern that allows for cell motility and extracellular matrix degradation during migration and invasion to an endothelial-like integrin profile of differentiated cells that form tight junctions in the arteries, creating high capacity, low resistance blood vessels that allow for the exchange of blood gasses and nutrients from the mother to the developing fetus [11,15,16]. Many of these same processes are conserved in mice [17] in that cells originating from a trophoblast (TB) stem cell progenitor migrate and invade the maternal arteries, but in mice, these are thought to derive from trophoblast giant cell intermediaries, rather than cytotrophoblast lineages [18].

Prior work in this lab uncovered the ankyrin repeat, SOCS box-containing 4 (ASB4) protein as a mediator of embryonic stem cell to endothelial differentiation [19]. Further, ASB4 was found to be highly expressed in the extraembryonic vasculature including the allantois, yolk sac, and most notably, the developing placenta [19]. Therefore, we decided to investigate the role of ASB4 in placental vascular development.

**ASB4 as a ubiquitin ligase**

ASB4 is an oxygen-sensitive E3 ligase that is a member of the Suppressor of Cytokine Signaling (SOCS) superfamily of proteins. All SOCS proteins share a constant C-terminal SOCS box domain and variable N-terminal protein-protein interaction motifs [20].
The SOCS box binds to an elongin-B/elongin-C/cullin/ROC complex of proteins whose function as components of a ubiquitin ligase complex has been well characterized [21]. The N-terminal protein interaction motif binds to substrate protein(s) to optimally position them for ubiquitination and subsequent degradation [22]. Thus, SOCS proteins are the substrate-receptors for an ubiquitin ligase complex that controls steady-state levels of substrate proteins. Because of this essential function, SOCS proteins like ASB4 are carefully regulated both at the transcriptional and post-translational level to tightly control substrate protein levels.

The central role of all E3 ligases is targeting substrate proteins for ubiquitination. The well characterized ubiquitination process involves three enzymes that catalyze the activation and transfer of the 7 kDa ubiquitin protein to the targeted substrate protein. Briefly, the E1 ubiquitin activating enzyme covalently binds to the ubiquitin protein via an ATP-dependent process then transfers it to the E2 ubiquitin conjugating enzyme. From there, the ubiquitin molecule is either transferred directly to the substrate protein, due to the resulting structural proximity modulated by the E3 ligase (where the E3 ligase never directly interacts with ubiquitin itself), or is step-wise transferred to the E3 ligase, and further conjugated to the substrate protein (Figure 1.2) [23].

There are three forms of ubiquitination: mono-, multi- (or multi-mono-), and polyubiquitination. Mono-ubiquitination is where a single ubiquitin moiety is conjugated to a single ε-NH₂ group of an internal lysine residue of the target substrate. Proteins can also undergo multi-ubiquitination, whereby a single ubiquitin is conjugated to multiple internal lysine residues on the target protein. Lastly, polyubiquitination occurs when multiple (typically greater than three) ubiquitin proteins are attached to a single internal lysine residue
(or a free α-\(\text{NH}_2\) group of the N-terminal residue) on the target substrate. These different ubiquitination reactions result in very different consequences for the substrate protein. Typically, mono- and multi-ubiquitination are thought to be involved in nonproteolytic, reversible, signaling events such as endocytosis, membrane trafficking, DNA repair, and gene silencing [24]. Polyubiquitination, in contrast, is generally thought of as being responsible for the proteasome-dependent degradation of a target protein. However, adding yet another layer of complexity to the ubiquitination process, ubiquitin chains can link to one of seven different lysine residues (K6, K11, K27, K29, K33, K48, and K63) within the ubiquitin molecule, leading to different signaling outcomes. Lysine 48-linked ubiquitin chains are by far the most widely studied and best characterized, and proteins with these K48 side chains are targeted for proteolysis [25]. K63-linked polyubiquitin chains instead lead to protein interactions that are involved in endocytic trafficking, inflammatory response, protein translation, and DNA repair [26]. The other five homotypic polyubiquitination chains (K6, K11, K27, K29 and K33) have been observed in cells; however, their roles are still emerging [27].

ASB4 is one of 18 proteins in the ASB family, which are part of the suppressors of cytokine signaling (SOCS) super-family. ASB4 contains nine ankyrin repeats, seven of which are highly conserved, and a C-terminal SOCS box [28]. Ankyrin repeats are common 33-residue motifs that mediate protein-protein interactions and are found in proteins with functions ranging from development to transcription and cell cycle control [29]. Like other members of the ASB family [30], ASB4 associates with cullin, elongin, and ROC/Rbx RING-finger proteins (possibly because ASB4 lacks a RING-finger domain), which are all part of the ubiquitin ligase complex [19]. There is little evidence indicating a central function
of ASB4. However, areas of high energy consumption (e.g., testes, heart, and brain) in adult mice have ASB4 ligase activity [31-33], reinforcing our hypothesis that ASB4 regulates vascular development and differentiation [34,35]. Further, ASB4 is abundantly expressed in the developing placenta and is highly upregulated during the differentiation of embryonic stem (ES) cells into endothelial cell lineages [19]. In addition, Asb4 transcription decreases when endothelial cells are challenged by laminar shear stress [36], highlighting the importance of ASB4 in the vasculature.

Interestingly, many of the few reports describing ASB4 illustrate the epigenetic regulation of Asb4, specifically as an imprinted gene [37-40]. In the case of Asb4, only the maternal allele is expressed, while the paternal allele is silenced. Though genomic imprinting has been found in all Eutheria, less than one percent of all genes are imprinted [41]. Though not thoroughly understood, imprinting is thought as of a result of an evolving “disagreement” between maternal and paternal genes over the allocation of maternal resources to offspring, known as the Genetic Conflict Theory, which was originally identified by David Haig over twenty years ago [42]. Briefly, this theory states that genes in offspring are predicted to demand more resources from the mother than the mother is selected to provide, and the optimal level of demand on maternal resources may differ for the two alleles depending on the parent of origin. The genetic outcome of this parental antagonism predicts preferential expression from one of the parental alleles [43,44].

Perhaps not surprisingly, most imprinted genes are highly expressed in embryonic, fetal, and early post-natal stages of development. One site of high imprinting activity is in trophoblast cells and is readily apparent due to the fact that many imprinted gene mouse knockout models exhibit altered placental development and function [45], with many of these
imprinted genes showing conserved activity between mouse and human placental function and growth [46]. In addition to morphological differences, recent evidence points toward the role of imprinted genes mediating the cellular response to stressors or environmental cues such as diet [47], alcohol [48], and superovulation [49]. Imprinted genes also mediate ion and nutrient transport within the placenta [50,51] as well as growth and vascular function [52,53]. These functions illustrate the importance of imprinting as a key modulator of placental development and outcome during gestation.

One of the key components to our investigation of ASB4 was the identification of a target substrate protein. Though there are assays that can elucidate specific ligase-substrate interactions (e.g. yeast-2 hybrid, co-immunoprecipitation, and the recently described tripartite TUBE/2D-DIGE/MS assay [54]), all have major caveats. Thus, we resorted to a candidate approach in our search for a substrate for ASB4. We isolated ID2, described below, as a potential factor due to; its expression in the placenta during development, its role in mediating vascular differentiation in the placenta, and its ability to be degraded by the proteasome in ubiquitin-dependant manner.

**ID family of proteins**

The four members of the ubiquitously expressed family of Inhibitor of DNA binding (ID) helix-loop-helix (HLH) proteins, ID1-ID4, function as dominant negative regulators of basic HLH (bHLH) transcriptional regulators that mediate cell lineage commitment, differentiation, proliferation, cell cycle control and senescence, metastasis, angiogenesis, apoptosis, and maintaining cell ‘stemness’ [55-58]. Typical HLH proteins mediate homo- and heterodimerization and contain a highly basic region adjacent to the HLH domain, which facilitates transcriptional regulation via DNA binding to the canonical E- or N-box sequences
in target genes [59]. However, ID proteins lack this basic domain and instead function by dimerization with transcription factors, namely members of the bHLH superfamily. The resultant ID-bHLH heterodimer is thus unable to bind to DNA or mediate transcription (Figure 1-3) [60]. Because bHLH proteins typically positively regulate differentiation though DNA binding, ID proteins are also colloquially referred to as “inhibitors of differentiation.” Further, there is significant, but not ubiquitous redundancy between the individual ID proteins [61-64], which is not surprising given that the HLH domains of the ID proteins share 70-80% amino acid sequence identity [65].

ID proteins are tightly regulated by E3 ligases [66-68]. Unlike most ubiquitin substrate proteins that are targeted for degradation, ID1 and ID2 are ubiquitinated on their respective N-terminal residues [67,69]. To date, approximately a dozen other known proteins undergo N-terminal ubiquitination [70], which differs from the N-end rule pathway. N-terminal ubiquitination is when ubiquitin modification occurs on the free α- NH₂ group of the N-terminal residue of the substrate protein, with substrate recognition likely involving a downstream or internal motif; in the case of ID proteins, this motif is a destructive box (D-box) element [68]. Conversely, in the N-end rule pathway, the N-terminal residue of the substrate protein serves at the recognition motif for modification, but the ubiquitin modification itself takes place on an ε-NH₂ group of an internal lysine residue [71]. While the majority of proteins that are targeted for N-terminal ubiquitination contain internal lysine residues, three (p14ARF, HPV-58 E7, and p16INK4a) are naturally occurring lysine-less proteins [72,73]. However, while the internal lysine residues are not essential for the degradation of these substrates of N-terminal ubiquitination, these internal residues may still play a modulating role. That is, while lysine-less mutants of these substrate proteins are still
ubiquitinated and degraded, this reaction is slowed by two to three fold compared with that of lysine-containing wild-type proteins [74,75].

ID2 is one of the better studied members of the ID protein family [76] and is involved in vascular events, including angiogenesis [77] and tumor cell migration and invasion [78]. Further, ID2 is a tightly regulated mediator of placental development and vascular differentiation [77,79,80]. Although ID2 is rapidly cleared via the proteasome [67], little is known about the specific ubiquitin ligases that regulate its expression. To date, only the anaphase-promoting complex/cyclosome-Cdh1 (APC/C(Cdh1)) has been identified as a ubiquitin-mediated regulator of ID2 expression [68]. APC/C(Cdh1) restrains axonal growth and controls axonal morphogenesis in post-mitotic neurons [81]. Conversely, ID2 mutants that are resistant to APC/C(Cdh1) enhance axonal growth and overcome myelin inhibitory signals to promote growth. However, through its proteolytic targeting of ID2, APC/C(Cdh1) permits the accumulation of the Nogo receptor, a key transducer of myelin and axonal inhibition mediated by bHLH transcriptional activation. Thus, APC/C(Cdh1) relieves the ID2-mediated repression of bHLH transcription factors, which repress axonal growth, and is vital for proper synaptic patterning [81]. Although these studies elegantly illuminate how E3 ligases control ID2 in post-mitotic cell morphogenesis and how ID2 interacts with bHLH transcription factors in vivo, there is little evidence for other E3 ligases that regulate ID2 expression.

However, for ID2 to be a bona fide target of ASB4 in the placenta, the transgenic over-expression of Id2 in mice would have to show some placental vascular defects. Indeed, in retinoblastoma (Rb) deficient mice, there is a concordant upregulation of Id2 expression. These mice display die embryonically due to abnormal trophoblast proliferation and
placental dysplasia [82]. However, when *Id2* is also deleted in these mice, this placental phenotype is abrogated [83], indicating that overexpression of ID2 induces abnormal placental development. Therefore, using ID2 as a known regulator of placental differentiation and vascularization, we examined the placenta in the context of *Asb4* expression for similar pathologies.

**Pre-eclampsia and pathologies of the placenta**

The central function of the placenta is to provide for an efficient and robust exchange of nutrients and oxygen from the maternal blood supply to the fetal blood and to eliminate waste products from the fetal blood supply back to the maternal blood. During development, the entire conceptus unit (the embryo, yolk sac, chorion, and allantois) must synchronize blood vessel formation. That is, shortly after the initiation of blood flow within the embryo, the placental vasculature must also be completely formed, including its connection to the maternal uterine arteries [84]. In normal placentation, the maternal and fetal vessels don’t intermingle and the blood supplies never mix; however, the vessels must be localized in such close proximity and arrangement to ensure adequate and constant exchange of nutrients, oxygen, and waste. Therefore, the placenta must coordinate rapid expansion and growth to ensure the adequate flow of nutrients and oxygen to the growing fetus, with precise and controlled morphogenesis and vascular patterning to guarantee that vessels are in the correct location and are properly developed in anticipation of a high rate of blood flow. These vessel patterning events require an immensely intricate degree of vascular networking and remodeling from the fetal umbilical cord and chorion through the outer layers of the decidua and myometrium to ensure the health of not only the developing fetus but also that of the
mother, who is also at risk for vascular diseases during blood vessel development (Figure 1-4).

As maternal-fetal blood flow proceeds, the oxygen levels of the developing embryo increase rapidly from hypoxia to relative normoxia, and vessels experience rapidly increasing shear stresses. All cells must transduce these environmental signals into appropriate developmental responses [85]. This sudden increase in hemodynamics and the increase in blood gas oxygen levels act as environmental cues that influence additional endothelial development [86]. That is, hypoxia typically serves as a cue for angiogenesis, recruiting new blood vessels by secreting growth factors that act specifically on vascular cells, which leads to the breakdown of the vessel wall and concomitant migration and proliferation of endothelial cells towards the ischemic tissue. In endothelium, HIF (hypoxia-inducible factors) transcription factors induces; transcription of erythropoietin (EPO) which stimulates blood cell formation; transcription and secretion of VEGF and FGF which stimulate endothelial migration towards hypoxic tissue; transcription of Flk1 and Flt1, which are VEGF receptors potentiated under hypoxic conditions; and transcription of myriad genes that are responsible for the immediate response to hypoxia and induce anaerobic respiration, allowing for energy production in the absence of oxygen-dependant oxidative phosphorylation [87]. However, the factor inhibiting HIF (FIH) is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor [88] in an oxygen dependant manner [89]. Shear stress also mediates vascular remodeling through the cytoskeletal remodeling, release of nitric oxide, activation of transcription factors, and mediating growth factor expression [90]. ASB4 is a well know target of FIH hydroxylation [19], and is also regulated by shear stress at the transcriptional level [36].
Thus, the importance of ASB4 as an oxygen and hemodynamic sensor becomes apparent in the context of vascular remodeling at key developmental time points [19,36,87].

Not surprisingly, when this complicated vascularization process goes awry, disorganization or malformation of the placenta can cause deleterious effects. Typically, most placental pathologies are due to either defects in differentiation, migration, and invasion or are vascular. Failure of the decidua and/or blood vessels to attach to or penetrate the maternal myometrium can result in complications such as abruptio placentae (placental separation from the uterus) and placenta accreta, increta, and percreta (the abnormally strong and advancing penetration into the uterus). Vascular defects in the myometrium, endometrium, and placenta result in myriad defects ranging from placenta praevia and chorangiosis to decidual and fetal vasculopathy [85]. Perhaps the most common placental pathology is pre-eclampsia, which affects roughly five percent of all pregnancies [91].

Pre-eclampsia is characterized as the sudden onset of maternal hypertension, proteinuria, and edema and typically occurs during the third trimester of pregnancy [92]. There is currently no cure for pre-eclampsia, other than delivery, which often happens prior to the 40-week term in humans. Currently, the only treatments to alleviate the symptoms of pre-eclampsia are antihypertensives, typically magnesium sulfate, which lower blood pressure and slow the heart rate [93]. If left untreated and in severe cases of pre-eclampsia, pre-eclampsia may progress into eclampsia (i.e., seizures) either during gestation or after delivery, resulting in a relatively high incidence of maternal and/or fetal death [94,95]. Other syndromes, such as HELLP (Hemolysis, Elevated Liver enzymes, Low Platelet count) and intrauterine growth restriction (IUGR) may also be associated with pre-eclampsia [96,97]. The resultant costs of pre-eclampsia and consequent morbidities, treatment, and life-long
effects are considerable, and the hospital treatment of hypertensive pregnancies alone is approximately $3 billion in the US annually [98].

The underlying pathophysiology of pre-eclampsia is thought to be rooted in vascular dysfunction [99]. Incomplete or dysmorphic maternal spiral artery remodeling, global endothelial cell dysfunction, and the aberrant reduction in placental vasculature are all hallmarks of pre-eclampsia.[100]. While probably multifactorial, this vascular insufficiency may be due to aberrant early TB differentiation [101]. In both humans and mice, vascular progenitor trophoblasts must differentiate, migrate, and invade to ensure proper neovascularization and vascular remodeling [102]. Some factors, such as placental growth factor (PIGF), vascular endothelial growth factor (VEGF), and soluble-Fms-like tyrosine kinase 1 (sFlt1) mediate the anti-angiogenic response [100], while other factors, such as human chorionic gonadotropin (hCG) and matrix metalloproteinases (MMPs), affect invasion and migration [103]. However, little is known about the initial differentiation events that ensure vascular lineage commitment from trophoblast stem cells.

Hypothesis

In our pursuit to elucidate factors that mediate early vascular development, we have identified ASB4 as an understudied E3 ligase that could potentially regulate extraembryonic vascular development. Likewise, we have identified ID2 as a potential candidate protein that may be regulated by ASB4, as ID2 mediates critical factors of placental differentiation and vascularization. However, many questions remain. Is Asb4 expressed in the trophoblast stem cells that have committed to a vascular lineage? Does the deletion of Asb4 in vivo induce changes in differentiation? Does ASB4 regulate ID2 directly, polyubiquitinating ID2 and targeting it for proteasomal degradation? Do ASB4 and ID2 have functional consequences in
placental cells? Does the deletion of \textit{Asb4} in mice induce a placental pathology? In the following chapter, we address our central hypothesis that ASB4 promotes trophoblast-to-endothelium differentiation through the degradation of ID2 by using a combination of \textit{in vitro} and \textit{in vivo} approaches.
Figure 1.1. Placental development and differentiation.
A. The inner cell mass (ICM) of the blastocyst consists of the stem cell population that will comprise the developing embryo, while the outer layer of trophectoderm (TE) will make up the placenta. (Image adapted from M. Hemberger, The Babraham Institute)

B. Trophoblast (TB) stem cells of the trophoblast layer commit to several lineages and cell types throughout endothelial differentiation within the placenta. TB stem cells can either form blood cell precursors (hematopoietic cells) or endothelial precursors (angioblasts). Angioblasts can then differentiate into perivascular cells (e.g. vascular smooth muscle cells)
or primitive cytotrophoblasts (CTBs, in humans) or primitive trophoblast giant cells (TGCs, in mice). These cells can then either form the epithelial syncytiotrophoblasts or the endothelial-like vascular CTBs and vascular TGCs that will form the vascular system of the placenta.

C. The placenta and corresponding fetal and maternal vasculature develops throughout gestation. The outer trophoblast layer of the conceptus unit (left) develops into the chorionic villi that circulates the fetal blood supply in close proximity to the maternal spiral arteries (right). The allantois is the primitive vascular structure that eventually forms the umbilical structure, while the yolk sac provides early circulatory function prior to the initiation of blood flow in the placenta. The junction of the fetal vascular network (dark purple) and the maternal arteries (pink) is highlighted on the right, demonstrating the compartmentalized, but “intertwined” network of the placental vasculature. (Image adapted from Chaper 34 of [104])
Figure 1.2. **ASB4 as an E3 ligase.**

A. ASB4 is a 426 amino acid protein with three identifiable regions represented in ribbon structure: an N-terminal variable domain (NTV), which functions in providing substrate specificity; nine ankyrin repeats (denoted by AR1-AR9) that mediate protein-protein interactions and protein folding; and a C-terminal suppressor of cytokine signaling (SOCS) box, which binds to adaptor proteins of the ubiquitin ligase complex.

B. Like other SOCS proteins, ASB4 binds an elongin-B/elongin-C/cullin/ROC1 complex through its SOCS box, which forms the components that mediate substrate protein ubiquitination. In this reaction, ubiquitin (U) is transferred from the ubiquitin activating enzyme (E1) to the ubiquitin conjugating enzyme (E2). Ubiquitin is either then transferred directly to the substrate (as shown) or is transferred to the E3 ligase prior to the substrate. This reaction repeats over several cycles, creating a polyubiquitin chain that targets the substrate protein to the proteasome for degradation.
Figure 1.3. **ID proteins regulates bHLH-mediated transcription.**
Cells are maintained in “State 1” by the helix-loop-helix (HLH) inhibitor of DNA binding (ID) proteins, which lack the basic DNA binding region that is common to the basic HLH transcription factor family members. The ubiquitously expressed basic helix-loop-helix (bHLH) E protein transcription factors activate transcription (State 2) by binding to promoter E boxes as E protein–E protein homodimers (not shown in figure) or as E protein–tissue-specific bHLH factor heterodimers. Formation of ID protein–E protein dimers prevents E proteins from forming DNA-binding transcriptionally active complexes. ID downregulation is necessary to allow for cell entry to “State 2”. (Image reprinted with permission from [105])
Figure 1.4. Vascular remodeling during placental development.

A. Spiral arteries from the maternal myometrium are remodeled during placental development, ensuring a high capacitance, low resistance blood flow to bathe the fetal circulation. As cytotrophoblasts (CTBs, in humans) or trophoblast giant cells (TGCs, in mice) differentiate, they migrate and invade from the fetal components of the placenta to the decidual space, and replace the endothelial cells of the spiral arteries. (Adapted from [106])

B. By the third trimester of normal placental development, invasive CTBs/TGCs of fetal origin have transformed spiral arteries them from small-caliber resistance vessels to high-caliber capacitance vessels capable of providing placental perfusion adequate to sustain the growing fetus. During the process of vascular invasion, the CTBs/TGCs differentiate from an epithelial phenotype to an endothelial phenotype, a process referred to as
"pseudovasculogenesis" (upper panel). In preeclampsia, CTBs/TGCs fail to adopt an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow and they remain small caliber, resistance vessels (lower panel), which may result in the placental ischemia, maternal hypertension, and fetal growth restriction. (Image reprinted with permission from [107])
CHAPTER 2

ASB4 PROMOTES TROPHOBLAST DIFFERENTIATION THROUGH THE DEGRADATION OF ID2

Introduction

Previous work in this laboratory demonstrated that ASB4 is an oxygen-sensitive E3 ligase that is abundantly expressed in the developing placenta and is highly upregulated during the differentiation of embryonic stem (ES) cells into endothelial cell lineages [19]. Also, ASB4 associates with cullin, elongin, and ROC/Rbx RING-finger proteins (possibly because ASB4 lacks a RING-finger domain), which are all part of the ubiquitin ligase complex [19]. Based on the high expression levels of Asb4 in the developing placenta, coinciding with the role of ASB4 in vascular differentiation [19], we reasoned that any putative substrates would share expression patterns and function within in the developing vasculature.

ID2, a part of the anti-differentiation ID protein family, is a tightly regulated mediator of placental development and vascular differentiation [77,79,80]. Due to the spatial and temporal overlap and the functional contrast between these two proteins, we hypothesized that ASB4 negatively regulates placental endothelial differentiation via and degradation of ID2.

In this Chapter, we investigated the role of ASB4 in TB cell differentiation and function and identified ID2 as a substrate of ASB4s ubiquitin ligase activity. Placentas isolated from Asb4−/− mice exhibited vascular differentiation defects, dysmorphic placental
vessels, vascular dysfunction, and spontaneous abortion in a subset of fetuses. Using cell culture models, we found that ASB4 directly interacted with ID2, leading to ID2’s ubiquitination and subsequent degradation in JAR cells. Further, ASB4 promoted aspects of placental cell differentiation and endothelial cell replacement and vessel stability. Co-transfecting Asb4 with Id2 mutants that are resistant to proteasomal degradation abolished these effects. Lastly, pregnant Asb4−/− mice exhibited symptoms consistent with pre-eclampsia, including proteinuria and hypertension.

**Material and Methods**

**Mouse generation, blood pressure, and proteinuria**

The Asb4+/− mouse generation is described by Ferguson [87]. Briefly, exon 1 of Asb4 was flanked by loxP excision sites in the pAMC vector. Positive recombinants were electroporated into 129 SvEv ES cells and cultured with appropriate selection enzymes. ES cells were then injected into C57Bl/6 blastocysts and implanted into pseudopregnant females. The resultant chimera (Asb40ox/+ ) was then mated with EIIa-cre mice to excise the loxP sites. These mice were further bred to 129 SvEv wild-type mice to ensure germ-line transmission of the deletion and to outbreed the cre allele, generating Asb4+/− mice on the 129 SvEv background.

Maternal blood pressure was measured in conscious, pregnant mice at the gestational stage indicated using a CODA 8 tail-cuff monitor (Kent Scientific, Torrington, CT, USA). Mice were habituated to the machine for one day prior to data collection and assayed for five consecutive days. Urinary creatinine and albumin protein levels were measured using the Creatinine Companion and Albuwell M Test kits, respectively (Exocell, Philadelphia, PA, USA). Urine collection consisted of placing isolated mice in metabolic cages (a generous gift
from Dr. Nobuyo Maeda [University of North Carolina]) for 24 hours. Food and water were provided ad libitum, and urine was collected in a microcentrifuge tube placed below the mesh flooring. Particulate matters and solids were removed from the samples by benchtop centrifugation, and urine was stored at -20°C until assayed. Placental disc invasion was assessed in E17.5 placentas as described in Dokras et al. [108]. All experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

In situ hybridization, immunofluorescence, and immunohistochemistry

In situ hybridization for Asb4 was performed by the UNC In Situ Hybridization Core Facility on 16-μm thick cryosections of placental tissue harvested under RNase-free conditions from E11.5 wild-type mice. To generate the Asb4 probe, a ~900 bp fragment of Asb4 was TA-cloned into pCRII-TOPO using the primers 5’-CTCCGAGGATGGACGGCATCACTGCCCCTATC-3’ and 5’-CTCAGGCTGTGCAGCAGGACGC-3’. The fragment was excised using NotI and BamHI restriction enzymes. Sense and anti-sense probes were generated by transcription with T7 and Sp6 polymerase, respectively. Probes were digoxigenin-labeled prior to hybridization.

Immunofluorescence and immunohistochemistry were performed as described in Waldo et al. [109] on placental tissue sections at the indicated embryonic day. Briefly, tissue was harvested and either flash frozen or fixed in 4% paraformaldehyde overnight with subsequent cryoprotection in 30% sucrose. Samples were embedded in OTC Compound (Sakura Finetek, Torrance, CA, USA) and sectioned into 6-μm thick slices by the UNC Histology Research Core Facility. Primary antibodies are as follows: antibody recognizing mouse ASB4 was generated as in Ferguson [87]; c-kit (Cell Signaling Technology, Danvers, MA, USA);
PECAM (Becton-Dickinson, San Jose, CA, USA); cytokeratin-17, integrin alpha V, and integrin beta 4 (Abcam); ID2 (Cell Signalling Technology); Von Willebrand factor (Dako, Carpinteria, CA, USA); FITC-conjugated Dolichos biflorus agglutinin (DBA) (Sigma-Aldrich, St. Louis, MO, USA); and phospho-histone 3 (Millipore, Billerica, MA, USA). Alexa Fluor antibodies (Invitrogen, Grand Island, NY, USA) and ABC Elite kits and diaminobenzidine (Vector Labs, Burlingame, CA, USA) were used to detect primary antibodies. Apoptosis was quantified using the ApopTag In Situ Apoptosis detection kit (Millipore). Hematoxylin and eosin staining was performed on fixed frozen sections by the UNC Histology Core Facility. Tissues and cells were imaged on a Nikon E800 upright fluorescent microscope, and ImageJ (http://rsbweb.nih.gov/ij/) was used for quantification and intensity measurements.

Cell Culture and Immunoblotting

JAR choriocarcinoma cells were obtained from ATCC (Manassas, VA, USA) and maintained in MEM supplemented with 10% FBS. HEK-293T/17 cells and 2H-11 endothelial cells were maintained in DMEM supplemented with 10% FBS. 2H-11 cells were conditioned to constitutively ectopically express ASB4 by transfecting cells with p3xFLAG-CMV10-Asb4, and stable clones were selected with G418 for 12 days. Transgene expression was confirmed by anti-FLAG immunoblotting. A stable Asb4 knockdown cell line was created in 2H-11 cells using mouse Asb4 shRNA lentiviral particles. Endogenous Asb4 expression was screened by reverse transcription-PCR. Transfection reactions were performed using LTX and Plus reagent (Invitrogen) according to Wolfe [110]. pCMV2B-Asb4 and p3xFLAG-CMV10-Asb4 were generated and used as described in Ferguson [87]. Id2-Sport6 and pCS2-Id2 mutants were generous gifts from Dr. Aaron Ciechanover
siRNA transfections were performed using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. The siAsb4 duplex sequence is as follows: 5’-CCACAAUGCUACCAUCAA-3’ and 5’-AGUUGAUGGUAGCAUUG-3’, and siRNA was synthesized and duplexed by Integrated DNA Technologies (Coralville, IA, USA). Cell lysis reactions were performed in cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing 1% Triton (immunoblots) or 0.5% NP-40 (immunoprecipitations). Cell fractionation assays were performed using NE-PER Nuclear and Cytoplasmic extraction kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s protocol. Cycloheximide was used at 50 µM in DMSO. Immunoprecipitation reactions were lysed as described above and crosslinked with 2 mM DSP (dithiobis(succinimidylpropionate)) (Thermo Scientific) for 2 hours at 4°C. Lysates were pre-cleared with the appropriate species IgG and Protein A/G beads (Santa Cruz Biotechnology) for 1 hour at 4°C and then incubated with either anti-c-myc or anti-FLAG affinity gel (Sigma-Aldrich). Primary antibodies include ID2 (Cell Signaling Technology); FLAG-HRP, myc-HRP, and GAPDH (Sigma-Aldrich); KDM1 and MEK1 (Abcam, Cambridge, MA, USA); and HA-HRP (Roche). Proteins were detected using HRP-conjugated, species-appropriate secondary antibodies (Sigma-Aldrich) and developed using TMA-6 reagents (Lumigen, Southfield, MA, USA).

In vitro ubiquitination assay

Recombinant ID2 was generated using an ID2-GST fusion construct (pGEX-2T-Id2) graciously supplied by Dr. Antonio Iavarone (Columbia University) in BL21(DE3)pLysS chemicompetent cells (Agilent Technologies, Santa Clara, CA, USA). Recombinant ASB4
was generated by ectopically expressing p3xFLAG-CMV10-Asb4 in HEK-293T/17 cells. E1 (Ube1), E2 (UbcH5a), ATP, and ubiquitin were purchased from Boston Biochem (Cambridge, MA, USA). The reaction buffer was as follows: 50 nM E1, 2.5 μM E2, 2.5 μM ASB4, 5 μM ID2, 2.5 mM ATP, 50 mM Tris, pH 7.5, 50 mM KCl, 0.2 mM DTT, and 250 μM ubiquitin. Reactions were performed at 37°C for 1 hour.

*Placental cell differentiation assays*

Trophoblast stem cells (TBSCs) were isolated as previously described [111] from E7.5 embryos. Cells were grown for 6-8 weeks under normal culture conditions to minimize spontaneous differentiation. Cells were cultured for 72 hours without serum and then visualized for the presence of trophoblast giant cells, a hallmark of TBSC differentiation in culture. JAR cells were induced to secrete human chorionic gonadotropin (hCG) using N(6),2′-O-dibutyryladenosine 3′:5′ cyclic monophosphate (dbcAMP, Sigma-Aldrich) at 1 mM for 24 hours as described in Hohn et al. [112]. hCG was measured using an ELISA (DRG International, Mountainside, NJ, USA), and concentrations were normalized to cell number. The JAR cell-mediated apoptosis of 2H-11 endothelial cells was evaluated based on Chen et al. [113], using the TUNEL-based ApopTag staining kit (Millipore) as an index of apoptosis. To assay endothelial network stability mediated by placental cells, we adapted the JAR cell/endothelial tube association assay from Aldo et al. [114], using 2H-11 endothelial cells as our vascular network substrate. JAR cells were transfected as indicated, then plated upon 2H-11 cells that had formed tube-like networks. Total area of the JAR/2H-11 network was measured using ImageJ and quantified.
Statistical analysis

Unless otherwise noted, statistical analysis for all quantification was performed using a two-tailed, unpaired Student’s t-Test. p values are reported in the respective figure legends.

Results

ASB4 is expressed in undifferentiated TB cells and is required for placental differentiation

Asb4 is localized to areas of high vascular activity and is highly expressed in the developing placenta [19,87]. ID2 is also critical in the early development of the placenta, including the maturation of the placental vasculature [77]. Therefore, we hypothesized that ASB4 would be an important modulator of differentiation in the placental vasculature. As shown in Figure 2.1A (4x magnification) and 2.1A’ (20x magnification), Asb4 mRNA was only expressed in the labyrinth zone of E11.5 placentas. This zone exhibits high vascular activity [115] and contains the reservoir of TB cells that cross the junctional zone into the maternal decidua as they mature into functional endothelial-like cells [15,116]. This observation supports our hypothesis that ASB4 is involved in early vascular differentiation events in the placenta.

To test whether ASB4 is expressed in differentiating cells committing to a vascular lineage, we examined placentas at E11.5, when stem cells undergo both self-renewal and differentiation and the cells adopting a vascular lineage migrate from the stem cell niche [117]. Wild-type placental tissue sections were co-labeled for ASB4 and markers of various stages of TB-to-endothelial differentiation. ASB4 co-localized with markers of pluripotent, cells, specifically in a subset of cells expressing the general stem cell marker c-kit (Figure 2.1B) [118]. At this stage in mouse placental development, invading and differentiating
trophoblasts express c-kit, but terminally differentiated trophoblast giant cells and spongiotrophoblasts do not. Further, c-kit and its ligand SCF are implicated as being required for trophoblast spreading and implantation, due to their role in trophoblast differentiation [119]. In addition, ASB4 also co-localized with a subset of PECAM-positive cells (Figure 2.1B’), suggesting that ASB4 is involved with cells that are differentiating into vascular lineages at this time point. Further supporting a specific role in early vascularization, ASB4 did not co-localize with cytokeratin 17, which is a marker of mature placental endothelial-like cells (Figure 2.1B”).

Because ASB4 co-localized with early markers of the endothelium, we hypothesized that Asb4 deletion would lead to functional consequences later in development. Specifically, if ASB4 promoted TB-to-endothelial cell differentiation, then placentas of Asb4<sup>−/−</sup> mice should have less mature endothelium than those of wild-type mice. Placentas from wild-type and Asb4<sup>−/−</sup> mice were examined at E17.5 and labeled for cytokeratin 17 (Cyto17) to visualize differentiated, mature endothelial-like cells. In wild-type mice, there abundant Cyto17 expression in the lining of the vessels, while in Asb4<sup>−/−</sup> mouse placentas there were fewer mature Cyto17-positive cells (Figure 2.2A), and this decrease was not due to impaired proliferation or increased apoptosis (Figure S2.1). During normal gestation, placental cells undergoing TB-to-endothelial differentiation undergo integrin “switching” [120], in which undifferentiated TB cells that adopt a vascular phenotype express beta 4 integrins. Later in gestation, when blood vessels have differentiated, beta 4 integrins are turned off, and alpha V integrin is highly expressed [117,121], consistent with what we see in our wild-type mice (Figure 2.2B, top panels). Asb4<sup>−/−</sup> placentas failed to express integrin alpha V, but maintained beta 4 integrin expression late in gestation (Figure 2.2B, bottom panels), consistent with a
failure of the placenta to undergo integrin switching, indicating an immature and undifferentiated placenta. Placental disc invasion, during which the fetal components of the placenta extend and expand into the maternal decidual layers, was more shallow in $Asb4^{-/-}$ placentas compared with wild-type mice (Figure 2.2C) further confirming that placental development is compromised in $Asb4^{-/-}$ mice in a manner that is consistent with abnormal differentiation [108]. Additional observations indicate that the vasculature expressed markers of injury and dysfunction in near-term (E17.5) $Asb4^{-/-}$ placentas (Figure S2.2A) and were mislocalized within the junctional zone rather than the stage-appropriate outer deciduas (Figures S2.2B and S2.2C). These results indicate that differentiation defects in $Asb4^{-/-}$ placentas may have deleterious effects that are observed into late gestation.

Because $Asb4^{-/-}$ placentas showed signs of early differentiation defects and impaired vascularization (Figures 2.2A, B), we examined ID2 expression due to its anti-differentiation role in the placenta [79]. We hypothesized that ID2 expression would be increased in the $Asb4^{-/-}$ placenta due to ID2’s anti-differentiation role in the placenta, and thus might underlie the observed differentiation defects in $Asb4^{-/-}$ placentas. In wild-type mice, ID2 expression is downregulated as TB cells differentiate. However, in whole placental cell lysates at E12.5, placentas from $Asb4^{-/-}$ mice have a ~2 fold increase in ID2 expression compared with placentas from wild-type mice (Figure 2.3A), and this finding is confirmed by immunofluorescence in E13.5 placentas (Figure 2.3B). These data indicate that a subset of TB cell remain undifferentiated in $Asb4^{-/-}$ placentas, and provide evidence that ASB4 may mediate ID2 expression in the placenta.
ASB4 negatively regulates ID2 expression through polyubiquitination and proteasome dependant degradation

Given our observation that ID2 was significantly upregulated in Asb4−/− placentas, we hypothesized that ID2 may be a substrate of ASB4s ubiquitin ligase activity. To test this hypothesis, wild-type Id2 was co-transfected with Asb4 in JAR cells, and ID2 expression was examined. JAR cells are a desirable cell type because they do not express endogenous ID2 or ASB4, allowing us to modulate both proteins without endogenous protein interference. Because ID2 is rapidly turned over by myriad other proteins, resulting in a very short half-life (Figure S2.4B and [67]), we added a low dose of MG-132 to experiments in Figure 2.4A-E to slow proteasomal degradation events and visualize ID2 protein expression. ASB4 degraded ID2 in a dose-dependent manner in Asb4 and Id2 co-transfected JAR cells (Figure 2.4A). To test whether ID2 expression increased in the absence of ASB4, we transfected Id2 into 2H-11 endothelial cells that stably overexpressed Asb4. ID2 expression increased when co-transfected with increasing amounts of an siRNA duplex targeting Asb4 (siAsb4) (Figure 2.4B). To determine whether ASB4 binds directly to ID2, we performed co-immunoprecipitation assays using co-transfected 3x-FLAG-tagged Asb4 and 6x-myc-tagged Id2 in JAR cells. As shown in Figure 2.4C, ASB4 was detected when ID2 was immunoprecipitated and, conversely, ID2 was detected when ASB4 was immunoprecipitated (Figure 2.4D). Further, we observed a dose response of the ID2-ASB4 interaction in 2H-11 cells that had increasing amounts of Id2 transfected in (Figure 2.4E). Together, these data show that in placental cells, ASB4 can mediate ID2 expression and that ASB4 and ID2 interact with each other.
Because ASB4 is an E3 ligase, we hypothesized that ASB4 regulates ID2 protein levels by polyubiquitinating ID2 and targeting it for proteasomal degradation. To test this hypothesis, we first co-transfected HA-tagged ubiquitin and myc-tagged Id2 in 2H-11 cells that either express endogenous Asb4 or have constitutively knocked down Asb4 expression to levels undetectable at either the transcript or protein level. We immunoprecipitated ID2 and blotted for HA, expecting a ubiquitin “smear” if ID2 was modified by polyubiquitination. As shown in Figure 2.4F, ID2 ubiquitination increased dramatically in cells that expressed Asb4, compared to cells that do not express Asb4. We then tested whether ASB4 could directly ubiquitinate ID2 by performing an in vitro ubiquitination assay. We combined recombinant ID2 with ASB4 and the minimal components required for ubiquitination and saw that ASB4 ubiquitinated ID2 four-fold more than the reaction absent of ASB4 (Figure 2.4G, lane 6 versus lane 3). Importantly, these reactions were performed in the absence of Roc1/Rbx1, the RING-finger protein that associates with ASB4, indicating that ASB4 does not require a RING-finger protein for ubiquitination. Previous reports into the mechanism of ID2 ubiquitination have demonstrated that ID2 is only susceptible to N-terminal ubiquitination [67]. To determine whether ID2’s N-terminus is sensitive to ASB4-mediated degradation, we co-expressed ASB4 with ID2 mutants that lack all lysine residues (LL-ID2) or have 6x-myc tags on either the N-terminus (degradation resistant, DR-ID2) or C-terminus (degradation sensitive, DS-ID2) in JAR cells. Only the N-terminally tagged ID2 (DR-ID2) expression level remained unchanged in the presence of ASB4 (Figure S2.3), indicating that ASB4 mediates ID2 degradation via N-terminal ubiquitination.

To confirm our hypothesis that ASB4 degrades ID2 in a proteasome-dependent manner, we completely abolished proteasomal activity with a high dose of MG-132 to cells.
ectopically expressing ID2 and either ASB4 or vector control and compared ID2 expression to cells that were not treated with MG-132. In cells treated with DMSO, ASB4 expression led to reduced expression of ID2 as in Figure 2.4A. While a high dose of MG-132 increased total ID2 expression in the absence of ASB4, this increase was not diminished by the co-expression of ASB4 in the presence of MG-132 indicating that ID2 is degraded via the proteasome (Figure S2.4A). Further, when cells ectopically expressing ASB4 and ID2 were treated with cycloheximide to block protein translation, the half-life of ID2 decreased in the presence of ASB4 (Figure S2.4B). To ensure that the reduction in ID2 expression in the soluble fraction assayed above was not caused by ASB4 inducing ID2 translocation to an insoluble part of the cell, we performed a cell fractionation assay (Figure S2.4C). There was no observable accumulation of ID2 in any of the cell fractions when co-expressed with ASB4, suggesting that ID2 is not translocated to other insoluble fractions of the cell upon treatment with ASB4.

**ASB4 mediates placental cell differentiation and function in vitro**

In culture, TB cells can induce endothelial turnover [113] and increase the stability of endothelial cell networks [114], recapitulating the in vivo events that occur when TB cells are differentiating into endothelial-like cells. Because Asb4−/− placentas express markers of undifferentiated TB cells (Figure 2.2), we hypothesized that ASB4 would increase the JAR cell-mediated apoptosis of 2H-11 endothelial cells as well as the vascular stability of vessel-like networks formed by 2H-11 cells in culture. We observed that JAR cells transfected with ASB4 induced 2H-11 endothelial cells to apoptose 3-fold more than vector control cells. Co-expression of wild-type ID2 did not attenuate apoptosis, but JAR cells co-expressing DR-ID2 and ASB4 resulted in significantly fewer TUNEL-positive 2H-11 cells compared with JAR
cells that were only transfected with ASB4. Of note, DR-ID2 and ASB4 co-expression elevated apoptosis compared with vector control cells, but this was significantly less than cells that did express ASB4 alone (Figure 2.5A, B). These data demonstrate that ASB4 promotes a functional vascular phenotype that recapitulates in vivo endothelial replacement with differentiating TB cells and that ID2 represses this effect.

Previous reports demonstrated that endothelial cells induce TB migration in culture and that TB cells stabilize these endothelial vascular networks [114], representing a model of the in vivo events that occur during TB differentiation [15]. To examine whether ASB4 could promote TB cell stabilization of endothelial cell networks, we measured the ability of JAR cells transfected with Asb4 and Id2 to form stable vascular networks over time, using branching 2H-11 tube-like structures as the strata upon which JAR cells could migrate to and stabilize. In isolation, 2H-11 cells plated on Matrigel consistently form branching tube-like structures within approximately four hours [122] but devolve into spheroid cluster of cells within 16 hours (data not shown). However, the addition of trophoblast cells can stabilize these networks for days and even weeks in culture [114]. Therefore, we hypothesized that Asb4-transfected JAR cells would stabilize these 2H-11 cell networks for significantly longer than vector-transfected JAR control cells. JAR cells expressing ASB4, either alone or co-transfected with wild-type Id2, were able to maintain and stabilize the 2H-11 vascular networks well past 16 hours, when vector control cell networks had destabilized (Figure 2.5C, D). Cells co-transfected with Asb4 and DR-Id2 quickly and significantly destabilized 2H-11 networks at 16 hours and were indistinguishable from control networks after 48 hours. These results indicate that ASB4 promotes TB endothelial-like cell function in vitro and that
ASB4 mediates these effects by degrading ID2, since DR-ID2 attenuates this ASB4-mediated effect in placental cells.

Because ASB4 mediates vascular differentiation in ES cells [19] and we have demonstrated that ASB4 negatively regulated the anti-differentiation protein ID2 (Figure 2.4A, B), we hypothesized that ASB4 would mediate placental cell differentiation through the regulation of ID2 and tested this hypothesis in vitro. First, TBSCs were isolated from the extraembryonic ectoderm of early post-implantation (E7.5) wild-type and Asb4−/− embryos and cultured on a feeder layer of mitotically inactivated MEFs, which promote the long-term maintenance and proliferation of undifferentiated stem cells [19]. Large-scale multipotent differentiation is expected for the first several passages, so cultures were grown 6-8 weeks prior to serum-withdrawal. Terminally differentiated cells were sub-cultured out, leaving only the undifferentiated embryoid bodies of TBSCs. Although the factors required for TBSC-to-endothelial transformation are not yet know, TBSCs readily differentiate into trophoblast giant cells (TGCs) [111]. Based on previous work from this laboratory [123], we used serum withdrawal to promote TBSC differentiation. Thus, we used the appearance of TGCs as an index of TBSC differentiation. After serum withdrawal for 72 hours, we visualized the isolated cells with bright-field microscopy. As shown in Figure 2.6A, wild-type TBSCs largely differentiated into large, multinucleated TGCs, which were morphologically very different from the small, clustered, undifferentiated TBSCs that form embryoid bodies as seen in Asb4−/− cells (right panel). Further, differentiated TGCs laid flat on the culture dish, while undifferentiated embryoid bodies had raised edges and appeared more convex on the culture dish, allowing for easy identification.
To determine whether ASB4’s influence on TB cell differentiation involves ID2, we examined human chorionic gonadotropin (hCG) secretion, a well-established marker of trophoblast differentiation [112], in JAR cells that ectopically express ASB4 and ID2. hCG secretion was stimulated via the addition of dbcAMP to the growth medium following the indicated transfection for 48 hours and was subsequently measured in the medium by ELISA. ASB4 stimulated hCG production approximately 2 fold compared with the vector control. Co-transfecting wild-type Id2 with ASB4 did not abolish hCG production, but co-transfection of Asb4 and DR-Id2 prevented hCG stimulation (Figure 2.6B). Together with data in Figure 2.5, these results illustrate that ASB4 promotes placental cell differentiation and function in vitro, and that ID2 mutants resistant to ASB4-mediated degradation can inhibit the differentiation and function of TB cells in vitro.

Asb4−/− mice phenocopy human patients with pre-eclampsia

Because our data indicate that ASB4 mediates placental cell differentiation and function (Figures 2.4 and 2.5), and that Asb4 deletion has negative consequences in the placental vasculature throughout development (Figure 2.2 and Figure S2.2), we investigated whether the placental abnormalities found in Asb4−/− mouse placentas contributed to the placenta-specific disease pre-eclampsia, whose pathogenesis may stem from abnormal placental vascular development [7]. Asb4−/− female mice produced significantly smaller litter sizes compared with wild-type female mice (Figure 2.7B) due to spontaneous abortion mid-gestation (Figure 2.7A). Similarly, Asb4−/+ breeding pairs produced non-Mendelian ratios of pups that were significantly skewed toward higher numbers of wild-type animals at the expense of Asb4−/− pups (Figure 2.7C). When investigating the source of lethality in the Asb4−/− pregnancies, we observed that fetal growth halted at approximately E10.5 to E11.5 in a
subset of $Asb4^{-}$ embryos. These embryos lacked functioning placental vascularization (Figure 2.7A, and data not shown), which may contribute to the abortion and fetal reabsorption seen in $Asb4^{-}$ embryos [5].

Because ID2 expression is elevated in trophoblast cells placentas of women with pre-eclampsia [79] and $Asb4^{-}$ mouse placentas (Figure 2.2D, E), combined with the vascular defects observed in $Asb4^{-}$ placentas (Figure 2.2A, Figure S2.2), we investigated whether our $Asb4^{-}$ mice shared traits with human patients with pre-eclampsia, which is widely believed to be a disease of the placental vasculature [101]. Two hallmarks of pre-eclampsia are maternal hypertension and proteinuria during late-stage pregnancy. Pregnant $Asb4^{-}$ female mice had increased blood pressure during late gestation (E14-term), as compared to both gestationally age-matched wild-type mice and $Asb4^{-}$ mice during the first week of gestation (Figure 2.7D). Further, pregnant $Asb4^{-}$ female mice had higher ratios of albumin:creatinine protein in their urine during late stage pregnancy than wild-type mice (Figure 2.7E). Together, these results suggest that $Asb4^{-}$ mice phenocopy human pre-eclampsia and may serve as a model for both early placental vascularization and human placental disease.

**Discussion**

Strict control over the vascular patterning of the placenta is critical for both maternal and fetal survival [124]. Aberrant differentiation events early in development negatively affect the later formation of the vasculature [15], but relatively little is known what drives early differentiation events. Although none of the limited data that identify putative substrates or functions of ASB4 support a central function for ASB4 *in vivo* [19,33,36,40,125], prior work from this laboratory has shown that ASB4 is involved in early vascular differentiation and is highly expressed in the developing placenta [19]. Therefore,
we utilized Asb4−/− mice, in conjunction with placenta-derived cells, to determine the function
of ASB4 during placental vascular differentiation. Consistent with our previous work [19],
we found that ASB4 is largely localized to the early endothelium in the placenta. We also
found that Asb4 deletion induces the expression of markers of undifferentiation in the
placenta, including the anti-differentiation protein ID2. Based on this data, along with the
expression pattern of various markers of TB cells and endothelial differentiation in Asb4−/−
placentas, we determined that ASB4 is involved in the earlier stages of differentiation events,
and the consequences of Asb4 deletion persist into later stages of gestation resulting in
insufficient placental vascularization.

Due to the limited information found in the literature, identifying a substrate of
ASB4s ligase activity was central to this investigation. Taking a candidate approach, we
reasoned that any ASB4 substrate would have to share its narrow spatiotemporal expression
pattern, contribute to vascular phenotypes, and be involved in differentiation. We determined
that the ID family of proteins would fulfill these criteria [56]. The ID proteins (ID1 to ID4)
are known to mediate differentiation and cell cycle control, which impact cell functions such
as metastasis, angiogenesis, apoptosis, and maintaining stemness [55-58]. Within these
processes, there is significant, but not ubiquitous redundancy between the individual ID
proteins [61-64]. Further, ID proteins, and ID2 specifically, are known to be tightly regulated
by E3 ligases [66-68]. Therefore, ID2 was chosen for investigation in this study due to its
involvement in differentiation [55], vascular development [126], and placental maturation
[79]. Using cell culture and biochemical techniques, we determined that ASB4 can directly
negatively regulate ID2 expression.
Placental remodeling requires three unique vascular events for proper function: TBSC differentiation, replacement of endothelium with trophoblast cells, and vascular stabilization to form high capacity vessels [15]. Possibly because the JAR cell line was isolated from CTB cells in choriocarcinomas, these cells can be induced to mimic in vivo cells under certain conditions. We adapted several methods to assess cell differentiation and function in culture, and whether ASB4 promoted these events through the inhibition of ID2. Although these methods do not completely recapitulate in vivo events, they collectively indicate that ASB4 has a pro-vascular differentiation function in placental cells. By ectopically expressing ASB4 in the JAR cells, we were able to determine that ASB4 promotes all three aspects of placental vascular remodeling. In addition, using isolated TBSCs, we were able to observe primary TB cell differentiation in culture. TBSCs that lack Asb4 remained in undifferentiated embryoid bodies, in contrast to wild-type TBSCs which differentiated into TGCs upon serum withdrawal. Because exact markers of TBSC and endothelial differentiation are not well defined in the placenta, future studies will be needed to more precisely address these differentiation events.

Using Asb4−/− mice as a model for ASB4 function in vivo, we explored the phenotypic consequences of Asb4 deletion, focusing particularly on the early placenta. Although the majority of Asb4−/− embryos survived to term, all had placentas with varying degrees of vascular dysfunction. Further, embryonic lethality occurred in a subset of Asb4−/− embryos at approximately E10.5 due to gross endothelial disruption in the placenta. Furthermore, placental vascular dysfunction in Asb4−/− placentas also had deleterious effects on pregnant mice, phenocopying women with pre-eclampsia. Though extremely common, little is known about the pathogenesis of pre-eclampsia [127] and to date there is no cure other than delivery
of the placenta. Both third trimester hypertension and proteinuria, hallmarks of pre-eclampsia, were recapitulated in Asb4−/− mothers. This disease state, in conjunction with the differentiation defects in the placenta of Asb4−/− mice, provides a unique model of early vascular development. Ultimately, this model of pre-eclampsia and vascular dysfunction may be used to investigate therapeutic strategies for treating pre-eclampsia and other diseases of the placenta.
Figure 2.1. ASB4 is expressed in the developing placental vasculature.
A) *Asb4* mRNA is expressed only in the labyrinth zone of developing placentas. *In situ* hybridization was performed on E11.5 placental sections and imaged with bright field microscopy. Wide-field (4x, A) and higher magnification (20x, A’) anti-sense (AS)-probed sections illustrate *Asb4* localized to the labyrinth zone. A sense probe was used as a negative control (A”).

B) ASB4 is expressed in a subset of c-kit-positive and PECAM-positive cells but not mature cytokeratin 17-expressing cells. E11.5 placental sections were probed with markers of stem cells (c-kit, B), endothelial cells (PECAM, B’), and differentiated TB cells (cytokeratin 17, B”).
B”) and fluorescently imaged at 20x magnification. These images were then merged to show co-localization. ASB4 only co-localizes with cells expressing c-kit and PECAM (arrows) but not cytokeratin 17 (filled arrows). There are also subsets of c-kit or PECAM positive cells that ASB4 did not co-localize at this stage (filled arrows).
Figure 2.2. *Asb4<sup>−/−</sup>* placentas express markers of undifferentiated vasculature and TB cells.

A) Placentas lacking *Asb4* have reduced cytokeratin 17 expression in near-term placentas. E17.5 placental sections from wild-type and *Asb4<sup>−/−</sup>* mice were labeled with cytokeratin 17 (cyto17), a marker of terminally differentiated endothelial-like TB cells. Blood vessels (BV) in *Asb4<sup>−/−</sup>* placentas display reduced cytokeratin 17 labeling compared with BVs in wild-type placentas.

B) Placentas from E15.5 wild-type and *Asb4<sup>−/−</sup>* placentas were labeled for integrin alpha V, a marker of mature, terminally differentiated TB cells and integrin beta 4, a marker of immature, undifferentiated TB cells. Wild-type placentas express alpha V but not beta 4 integrins. Cells in *Asb4<sup>−/−</sup>* placentas retain integrin beta 4 expression and fail to express integrin alpha V.

C) Placental disc invasion is reduced in *Asb4<sup>−/−</sup>* mothers at E17.5, indicating restricted trophoblast expansion. The ratio of the placental disc (P) to the total placenta (the sum of the decidua (De) and the placental disc) is decreased in *Asb4<sup>−/−</sup>* placentas compared to wild-type placentas, indicating a defect in TB cell invasion and migration.
Figure 2.3. ID2 expression increases in placentas that lack Asb4.
A) Lysates from three E13.5 wild-type and Asb4<sup>−/−</sup> placentas were immunoblotted against ID2 (top panel, asterisk denotes nonspecific band) and quantified (bottom panel). p<0.01. JAR-WCL = whole cell lysates transfected with Id2 or vector and run as a positive immunoblotting control.
B) E12.5 sections from wild-type and Asb4<sup>−/−</sup> placentas were labeled for ID2 (left panel), confirming that wild-type TB cells at this stage have low ID2 expression while ID2 expression is dramatically greater in Asb4<sup>−/−</sup> placentas. 100x magnification.
Figure 2.4. ASB4 negatively regulates ID2 expression through polyubiquitination and associates with ID2 in JAR cells.
A) ASB4 represses ID2 expression in a dose-dependent manner. Wild-type Id2 and vector, 0.5, or 2 µg of Asb4 were co-transfected in JAR cells. ID2 expression decreases as the ASB4 expression increases.
B) ID2 expression increases as ASB4 expression decreases. 2H-11 cells that constitutively express high levels of ectopic ASB4 were transfected with Id2 and either a scrambled nucleotide siRNA duplex (siScr) or increasing doses (0.15 nM, 0.5 nM) of siAsb4 duplex. As ASB4 expression decreases, ID2 expression concurrently increases.
C and D) ID2 and ASB4 associate in JAR cells. 3xFLAG-tagged Asb4 and 6xmyc-tagged Id2 were co-transfected in JAR cells. Lysates were pre-cleared with species-specific IgG and Protein A/G agarose beads were run with these reactions as a control against non-specific binding. Pre-cleared lysates were either immunoprecipitated with anti-myc- or anti-FLAG-conjugated agarose beads and blotted for FLAG or myc, respectively (C, D). Gels were stained with coomassie post-transfer as a loading control for immunoprecipitations. Input represents 2.5% of total lysate. ASB4 is detected in ID2 immunoprecipitation; conversely, ID2 is detected with ASB4 immunoprecipitation.
E) 2H-11 cells that stably express FLAG-tagged Asb4 were transfected with increasing amounts of myc-tagged Id2. Cells were lysed and pre-cleared as in C and D, then immunoprecipitated with anti-myc conjugated agarose beads and then blotted for FLAG. FLAG expression increases in parallel with myc expression, indicating specific interaction between ID2 and ASB4.

F) ID2 ubiquitination increases in cells with ASB4 expression. Wild-type Id2 and HA-tagged ubiquitin were transfected into either 2H-11 cells that express endogenous Asb4 or 2H-11 cells that have Asb4 constitutively knocked down. ID2 was immunoprecipitated using anti-ID2 and then blotted against HA. Reactions were blotted on the same membrane. Input represents 2.5% of total lysate. Ubiquitination of ID2 increases in endothelial cells that express ASB4 compared with cells that do not.

G) ASB4 directly ubiquitinates ID2 in vitro. Recombinant ID2 was incubated with recombinant ASB4, and components of the reaction as indicated. Reactions were resolved on SDS-PAGE gels and immunoblotted against ID2. ID2 is ubiquitinated approximately four-fold more with ASB4 than without (lane 3). Quantification of ubiquitination is fold change relative to lane 5 (without ID2).
Figure 2.5. ASB4 promotes JAR cell-mediated endothelial apoptosis and stabilization of endothelial cell networks.
A) JAR cells expressing ASB4 promote 2H-11 cell apoptosis. JAR cells were transfected with vector, Asb4, Asb4 and wild-type Id2, or Asb4 and DR-Id2 prior to being seeded on top of 2H-11 monolayers.
B) TUNEL-positive cells were counted and are presented as the percent of total endothelial cells within the field. Asb4-transfected cells increase apoptosis of the underlying endothelial cells, even when transfected with wild-type Id2. DR-Id2 co-transfected with Asb4 inhibits JAR-mediated 2H-11 apoptosis. * p<0.01 as compared to vector/vector. † p<0.01 compared to Asb4-only transfection.
C) JAR cells transfected with Asb4 promote endothelial tube stability. 2H-11 cells were placed on Matrigel and allowed to form tube-like networks. JAR cells transfected as in A were then plated on the networks, and total network area was measured at the times indicated.
D) JAR cells expressing DR-ID2 destabilize 2H-11 cell networks at 16 hours, while cells expressing ASB4 or ASB4 and wild-type ID2 maintained the size of these 2H-11 cell networks compared to vector transfected cells at 48 hours after plating.* p<0.05, *** p<0.01 as compared to vector/vector.
Figure 2.6. ASB4 promotes TB cell differentiation \textit{in vitro.}
A) TB stem cells (TBSCs) were isolated from wild-type and \textit{Asb4}\textsuperscript{-/-} extraembryonic ectoderm at E7.5. Cells isolated from each conceptus were cultured in isolation, and these data represent 4 unique populations of cells for each genotype. Serum withdrawal induces the formation of large, multinucleated trophoblast giant cells (TGCs, arrows) that differentiate from TBSCs (asterisks). As shown, wild-type TBSCs largely differentiate into TGCs (left panel) while \textit{Asb4}\textsuperscript{-/-} cells remain in undifferentiated embryoid bodies (right panel). MEF-feeder cells are indicated by filled arrows. Dashed outlines indicate the border of non-MEF cell clusters.
B) JAR cells were transfected to express vector, \textit{Asb4}, or \textit{Asb4} co-transfected with vector, wild-type \textit{Id2}, or degradation-resistant \textit{Id2} (DR-\textit{Id2}). ASB4 induced hCG secretion, and co-expression of wild-type ID2 with ASB4 did not change hCG secretion compared to ASB4 expression alone. DR-ID2 prevented dcbAMP-induced hGC section, with concentrations of hCG no different than vector/vector transfected cells. * p<0.01 compared with vector/vector.
Figure 2.7. Pregnant Asb4−/− mice display symptoms of pre-eclampsia.
A) A subset of Asb4−/− embryos dies in utero. Asb4−/− littermates are shown at E12.5, illustrating the lack of placental vasculature and dramatically reduced fetal growth in a subset of Asb4−/− embryos.
B) Quantification of the average litter size of wildtype mice compared to Asb4−/− mice, taken from more than 25 litters from each group.
C) Heterozygous breeding results in a lower than expected number of Asb4−/− pups (p < 0.01, Fisher’s exact test).
D) Pregnant Asb4−/− mice have significantly elevated mean blood pressure in the third trimester of pregnancy compared with both Asb4−/− mice in the first week of pregnancy and wild-type mice in the third week of pregnancy. * p<0.01.
E) Pregnant Asb4−/− mice have significantly elevated urine-albumin:urine-creatinine in the third trimester of pregnancy compared with both non-pregnant Asb4−/− mice and wild-type mice in the third week of pregnancy. * p<0.01.
Figure S2.1. Diminished mature vasculature in Asb4<sup>−/−</sup> placentas is not due to increased apoptosis or abnormal proliferation.

E15.5 placental sections from wild-type and Asb4<sup>−/−</sup> mice were evaluated for aberrant proliferation or apoptosis using phospho-histone H3 (pH3) or TUNEL, respectively. In both cases, no discernible differences were noted between genotypes. BV; blood vessel. Arrows denote TUNEL-positive cells.
Figure S2.2. Asb4 deletion induces vascular dysfunction and mislocalization of blood vessels in the placenta.
A) Near-term (E17.5) placental sections were harvested and labeled with von Willibrand factor to measure thrombus response and DBA to determine uterine natural killer cell response. Asb4−/− placentas display elevated thrombus/thrombosis response (left panel) compared with wild-type placentas, indicating damaged vasculature. Further, there is a dramatic increase in activated uterine natural killer cells (right panel) in Asb4−/− tissues, indicating elevated macrophage and immune response, compared to wild-type tissue.
B) E17.5 placental sections were stained with hematoxylin and eosin and examined for gross morphology. Blood vessels (arrows) were counted and classified based on their location in the labyrinth (LZ), junctional (JZ), or decidual (DE) zones. Blood vessels in wild-type placentas are seen at the edge of the decidua in, whereas significantly more vessels in Asb4−/− placentas are located in the junctional zone, at the expense of the decidual zone.
C) Quantification of placental vessels in each respective zone, indicating that vascular invasion/migration is defective in the absence of Asb4. * p<0.01 compared to wild-type.
Figure S2.3. N-terminally tagged ID2 is resistant to ASB4-mediated degradation. A) JAR cells were transfected with wild-type Id2, Id2 lacking all lysine residues (LL-Id2), or Id2 with 6xMyc tags on either the N-terminus (DR-Id2) or the C-terminus (DS-Id2) in the absence or presence of ASB4. ASB4 is unable to degrade DR-ID2 but can efficiently degrade other ID2 mutants, which is quantified in (B). * p<0.01 compared to wild-type ID2.
Figure S2.4. ASB4 degrades ID2 in a proteasome-dependant manner, and does not affect ID2 cellular location.

A) JAR cells co-transfected with *Id2* and either vector or wild-type *Asb4* were treated with DMSO or MG-132. While overall ID2 expression increases in the presence of MG-132, ID2 expression decreases only in the presence of ASB4 in DMSO-treated cells, suggesting that ID2 is sensitive to proteasomal degradation when co-expressed with ASB4.

B) JAR cells were transfected as in A, then treated with cycloheximide for the indicated times. In the presence of ASB4 (right panel above, dashed line and open diamonds in graph), ID2 half-life is shortened from 40.2 minutes to 33 minutes compared to cells that only express ID2 (left panel above, solid line and solid boxes in graph) indicating that ASB4 mediates ID2 protein expression.
C) ID2 sub-cellular localization is not altered in the presence of ASB4. JAR cells transfected with Id2 and either vector or wild-type Asb4 were fractionated into the whole cell lysate (WCL), cytoplasmic (Cyto), nuclear (Nuc), and Triton-insoluble pellet (Pel) fractions. In all fractions, ID2 expression decreases in the presence of ASB4.
CHAPTER 3
GENERAL DISCUSSION

Placental development is a critical, but poorly understood process. Previous work indicated that ASB4 mediated early vascular differentiation, and was localized to the placenta. Thus I sought to fully characterize the role of ASB4 in placental vascularization and trophoblast differentiation.

The experiments described herein attempt to fully characterize the role of ASB4 during placental vascular development by uncovering its expression, molecular function, biologic consequences, and molecular regulation. Data generated here reveal that 1) Asb4 is expressed in pluripotent trophoblast cells committed to the vasculature during a narrow time window during differentiation; 2) ASB4, as a ubiquitination complex, degrades the HLH protein ID2; and promotes differentiation of the vascular lineage through, in part, its degradation of ID2; and 4) deletion of Asb4 in mice induces a placental vascular phenotype and recapitulates pre-eclampsia in Asb4−/− mothers (Chapter 2).

The role of ASB4 in vascular commitment and patterning

Previous investigations in this laboratory elucidated a role for ASB4 in the lineage commitment step of pluripotent-to-endothelial differentiation [19]. Further, due to the high expression pattern of Asb4 in “hotbeds” of vascular activity and vasculogenesis (e.g. allantois, yolk sac, etc.) [19], we hypothesized that ASB4 would be responsible, in part, for endothelial differentiation in these areas.
Earlier work from our laboratory on ASB4 function [19,36] led us to examine vascular development in the placenta. Ferguson, et al [19] described how Asb4 expression was restricted to a narrow spatiotemporal window in which the placenta was developing. This study also elucidated that ASB4 functioned as an oxygen sensor, further implicating its function in the placenta, where hemodynamic stresses and hypoxia/normoxia events are required for proper vascularization. Likewise, Bode, et al. [36] described how laminar shear stress repressed Asb4 expression. We therefore examined the gross morphological and physiological consequences of globally deleting Asb4 expression in mice, which was generated in [87].

Our initial investigation of Asb4−/− mice established that there was embryonic lethality in a subset of pups that was occurring at or about E9.5 (Figure 2.7A). This spontaneous abortion appeared to occur in the absence of any gross fetal defect or pathology, but the placentas of said pups resigned to intrauterine demise were dramatically less vascular or developed (Figure 2.7A, lower conceptus) compared with littermates that appeared to be morphologically “normal” (Figure 2.7A, upper conceptus). To begin understanding this pathology, a histological examination was undertaken and revealed that ASB4 expression only coincided with cells that were either pluripotent (Figure 2.1B) or cells that had committed to the vascular lineage (Figure 2.1B’). Cells that had fully differentiated into endothelium (Figure 2.1B”) and cells of different lineages (spongiotrophoblasts, trophoblast giant cells, syncytiotrophoblasts, etc.) did not express Asb4.

Though there have been recent advances in understanding mouse placental development, the field woefully lacks a definitive and authoritative consensus with regard to markers of differentiating cells. Therefore, there are some caveats in trying to infer specific
cell types through the use of protein expression markers in the mouse placenta. To identify pluripotent/stem cells in the context of the mouse placenta, we relied on the receptor c-Kit (also known as CD117 or mast/stem cell growth factor receptor [SCFR]). c-Kit is a receptor tyrosine kinase that binds to stem cell factor ligand and mediates proliferation, differentiation, and serves as a marker of progenitor cells of mature endothelium [128]. However, c-kit expression is also found in trophoblasts that invade, migrate, and differentiate into the TGC lineage [118,119], indicating that it may also serve as a marker undifferentiated CTBs and TGCs, though this expression pattern has not been empirically determined.

Similarly, the use platelet endothelial cell adhesion molecule (PECAM) (also known as CD31) and cytokeratin 17 for the determination of endothelial committed and mature endothelial-like cells, respectively, was based on studies investigating their roles in human placental tissue [129,130]. While we are confident that there are few or no discrepant functions of these broad endothelial specific molecules between mice and humans, we must acknowledge that there is a dearth of data that definitively describe these protein markers in differentiating mouse trophoblasts.

These experiments illuminate the role of ASB4 in placental vascular determination and differentiation. While we are limited by the scope of markers of endothelial differentiation in the placenta, we are convinced that ASB4 plays an important role in early placental vascular events, which correspond with earlier reports on the function of ASB4 [19]. However, to fully understand the role of ASB4, we asked how ASB4, as a ubiquitin ligase, would function in the developing endothelium, and its context in the placenta.
ASB4 as an E3 ligase

Like all E3 ubiquitin ligase proteins, the functional ramifications of ASB4 are dependent upon the function of the substrate protein(s) that it degrades. Multiple attempts to identify binding partners of ASB4 have not yielded the identification of ASB4 ubiquitination substrate proteins. Through the use of yeast 2-hybrid, the Patterson laboratory identified FIH as an interaction partner of ASB4, though no putative substrate proteins were identified [19]. This result not only highlights the limitations of this assay, but also that of identifying substrate proteins in general. The use of co-immunoprecipitation has also been successfully utilized to identify co-factors of ASB4 [19], but protein turn-over, lack of specificity, and difficulty in separating signal-to-noise all confound any results indicating a putative ASB4 target (data not shown and [87]).

Therefore, we took a candidate approach in our investigation into ASB4 substrates. We knew that any substrate of ASB4 must be involved in early differentiation of the vasculature, be expressed in CTBs in vivo, and have overexpression models that mimic Asb4 deletion. Literature searches and coinciding data (Figure 2.3B) indicated that members of the ID family would fit these criteria. Specifically, studies of the retinoblastoma (Rb) protein in mice indicate that Rb-deficient mice die embryonically due to abnormal trophoblast proliferation and placental dysplasia [82]. This effect, however, is ameliorated by the concomitant deletion of Id2 [83], signifying that Rb-mediated overexpression of ID2 induces abnormal placental development. This Rb-induced ID2 overexpression also leads to the formation of embryonal cancer, abnormal proliferation, and impaired angiogenesis, further highlighting the importance of ID2 in early vascular and differentiation/proliferation events [77].
Because ID2 mediates differentiation, invasion, and migration in CTBs [79], we hypothesized that ASB4 normally regulates ID2 via its role as an E3 ligase. The role of ID2 in human placental differentiation has served as the crux for our further investigation in our Asb4−/− mice. Specifically, in cells driven to overexpress ID2, invasion was abrogated, migration was abnormally enhanced, and these cells retained characteristics of undifferentiated cells. Also of note, the abnormal maintenance of ID2 expression throughout placental development was associated with placentas from pre-eclamptic patients. Taken together, this report specifies the role of ID2 in the context of placental differentiation and pre-eclampsia [79]. Other studies have also illuminated the importance of ID2 in the context of proper placental development. Specifically, ID2 mediates transcriptional activity in the placenta [80]; ID2 inhibits extravillous trophoblast-to-TGC differentiation [131]; and Id2 is expressed and involved in trophoblast stem cell differentiation [132-134]. In conjunction with the known expression profile of Asb4, the hypothesized function of ASB4, the consequence of Asb4 deletion in mice, and our data demonstrating that ID2 and ASB4 have reciprocal expression patterns (Figure 2.3B), we hypothesized that ID2 may be one substrate of ASB4.

In undertaking our biochemical assays measuring the interaction of ASB4 and ID2, we had to make several concessions. Foremost, we wanted to use the most representative cell line but had to concede that no placental cell line endogenously expresses both Asb4 and Id2. The CTB-derived JAR choriocarcinoma cell line best combined placental pertinence, ease of manipulation, and assay-to-assay utility [135]. Because our JAR cells lack endogenous Asb4 expression, we also utilized the 2h-11 endothelial cell line for our experiments that required
endogenous Asb4 expression. Because these 2h-11 cells are not placental in origin, these cells were limited to biochemical assays and assays that required endothelial cells [122].

ID2 has been shown to translocate from the nucleus to the cytoplasm during nervous cell differentiation [136,137]. However, we observed a predominance of nuclear ID2 expression in vivo, as has been observed elsewhere [138], though nuclear and cytoplasmic expression appears to be dependent on the differentiative and proliferative context of the cells, such as in models of cancer [139,140]. We did not see any shifting of ID2 expression in our JAR cell fractionation assay (Figure S2.4C), though we did not examine the expression pattern during differentiation. Thus, while we show that ID2 expression decreases in the presence of ASB4 in both the cytoplasm and nucleus, we did not explicitly demonstrate that this was due to ubiquitination. That is, we cannot exclude the possibility that ASB4 may somehow simply repress (or accentuate) translocation, which in turn may mediate ID2 stability. Similarly, we did not test whether ASB4 itself is able to translocation between the cytoplasm and nucleus as is the case of another ID2 ubiquitin ligase, APC/C-Cdh1 [141]. These translocation events will have to be demonstrated in future studies to complete the

Pseudovasculogenesis and placental vascular remodeling occurs over time in a complex three-dimensional space. Thus, to recapitulate these dynamic events, we adapted various protocols to assay these complex and multifaceted in vivo events using in culture techniques. By adapting various protocols, we were able to assay placental stem cell and CTB differentiation [111,112], endothelial cell replacement by CTBs [113], and vascular stability[114].

These assays and the resulting data are the best current techniques for addressing complex questions regarding placental development; however, certain limitations are
obvious. Trophoblast stem cell and CTB differentiation assays either occur in isolation (Figure 2.6A) or with exogenous pharmacological intervention (Figure 2.6B). Unlike true endothelial differentiation [123], the endogenous factors required for trophoblast-to-endothelial cell differentiation remain unknown. The models of CTB-mediated endothelial cell apoptosis (Figure 2.5A) and vascular stability (Figure 2.5C) also occur in the absence of exogenous factors, save for the components found in standard growth medium. However, these assays represent the best current techniques available for recreating in vivo placental defects and pathologies in cell culture.

**The role of ASB4 in placental pathologies and pre-eclampsia**

ASB4 is expressed in the developing placenta when the maturing vasculature is exposed to dynamic changes in environmental cues and undergoes drastic morphologic changes. Blood flow is initiated, which exerts hemodynamic 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 placental vasculature undergoes extensive changes during this time, including differentiating and making a vascular commitment, migration, and invasion. Links between these environmental cues and morphologic changes are just beginning to be identified in both normal and abnormal placental development, and ASB4 is spatiotemporally expressed and regulated in such a way as to represent a potential direct link between differentiation cues and vascular patterning responses.

The data presented in Chapter 2 suggest that ASB4 mediates the early differentiation steps that promote trophoblast cells into the vascular lineage, but does not exclude the possibility that ASB4 may also be involved in other vascular processes within the placenta.
Preliminary data in this laboratory indicate that ASB4 may mediate other aspects of vascular function, such as migration (via the Boyden Chamber and wound healing assay) and angiogenesis (via endothelial tube formation and subcutaneous Matrigel/tumor injections). While these data do not negate the effects we observe, they add to the possibility and plausibility that ASB4 functions in different contexts and milieus throughout development and adulthood.

Though many placental pathologies are not completely idiopathic, the etiology of most remains cloudy at best. As discussed in Chapter 1, disorders of placental adherence or invasion (e.g., abruption placentae, placenta accreta/increta/percreta) are often attributed to general vascular or blood dysfunction in cases where no previous or obvious trauma has occurred [142]. Disorders where the placenta blocks the ostium of uterus (i.e., external opening of the cervix) like placenta previa are again attributed to either previous trauma, or general vascular dysfunction/dysmorphia [143]. Lastly, common disorders such as pre-eclampsia, IUGR, and HELLP syndromes are all attributed to impaired differentiation and function of the early placental vasculature [96,97,101]. Almost all cases of dysfunctional, dysmorphic, and developmentally impaired placentas share some underlying vascular component to the etiology or consequence of the pathologies.

Though most medical technologies have made enormous strides in the past decades, the diagnosis and treatment for placental pathologies has remained woefully inadequate. For the vast majority of placental disorders in which comorbidities are present, early delivery remains the only option. The effects and consequences on both the mother and baby can be chronic and severe. Children delivered before term can suffer from broad respiratory illnesses (asthma, bronchopulmonary dysplasia), neurological delay or disorders (cerebral
palsy, autism), and physical ailments (enterocolitis, hearing loss, retinopathy) [144]. The cost to the mother can be just as great, resulting in complications from caesarean section, hysterectomy, eclampsia, gestational diabetes, hypertension, and infertility [142, 145, 146].

Diagnostically, placental pathologies remain critically understudied, and can currently only be diagnosed after they have developed, precluding early interventions. Typically, only the onset of sudden idiopathic and localized abdominal pain, edema, and dramatically increased blood pressure are used as indices for placental pathology. Further investigation using ultrasounds, elevated serum levels of alpha-fetoprotein or sFlt or Endoglin, and the onset of proteinurea are the only tools commonly available for diagnosis, and even then, most placental complications are little more than educated guesses. Early diagnostic biomarkers are critically needed.

Perhaps confounding this research is the fact that many placental pathologies do not present until late in gestation. Many patients appear asymptomatic until the third trimester, or even delivery, where complications can present fatal consequences if they are not prepared for. Our elucidation of ASB4’s function in early second trimester placental development may set a precedent for the earlier detection of pre-eclampsia, which may allow early interventional strategies that would prevent serious late-term complications. While the genetic component and contribution of ASB4 in human placental pathologies remains to be studied, there are many homologies between mouse and human placental development [147]. Thus, further studies that address the role of ASB4 in human placental pathologies will be of critical importance for the health of future mothers and infants.
Figure 3.1. Model of ASB4-mediated degradation of ID2 in trophoblast cells.
A) In the presence of ASB4 during gestation, ID2 is degraded and targeted for proteasomal degradation, allowing bHLH and E-protein transcription factors to bind to the E-box and promote pro-differentiation gene transcription, and ensures proper placental vascular development.
B) In the absence of ASB4 during pregnancy, ID2 negatively regulates E-protein and bHLH transcription factor activity. This abrogates trophoblast cell differentiation into the placental vasculature, and induces a maternal pre-eclamptic phenotype and fetal growth restriction.
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