Corrigendum

Mouse amnionless, which is required for primitive streak assembly, mediates cell-surface localization and endocytic function of cubulin on visceral endoderm and kidney proximal tubules

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The reference Fyfe et al. (2004) was listed incorrectly in the References.

The correct reference is:

The authors apologise to readers for this mistake.
Mouse amnionless, which is required for primitive streak assembly, mediates cell-surface localization and endocytic function of cubilin on visceral endoderm and kidney proximal tubules

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Summary

Impaired primitive streak assembly in the mouse amnionless (ann) mutant results in the absence of non-axial trunk mesoderm, a derivative of the middle region of the primitive streak. In addition, the epiblast of ann mutants fails to increase significantly in size after E7.0, indicating that middle primitive streak assembly is mechanistically tied to the growth of the embryo during gastrulation. Ann, a novel transmembrane protein, is expressed exclusively in an extra-embryonic tissue, visceral endoderm (VE), during the early post-implantation stages. We show that Ann is also expressed in kidney proximal tubules (KPT) and intestinal epithelium, which, like the VE, are polarized epithelia specialized for resorption and secretion. To explore whether Ann participates in the development or function of KPT and intestinal epithelia and to gain insight into the function of Ann during gastrulation, we constructed Ann+/− ES cell+/+/+ blastocyst chimeras. While chimeras form anatomically normal kidneys and intestine, they exhibit variable, selective proteinuria, a sign of KPT malfunction. In humans, AMN has been genetically connected to Cubilin (CUBN), a multi-ligand scavenger receptor expressed by KPT, intestine and yolk sac. Loss of CUBN, the intestinal intrinsic factor (IF)-vitamin B12 receptor, results in hereditary megaloblastic anemia (MGA1), owing to vitamin B12 malabsorption. The recent report of MGA1 families with mutations in AMN suggests that AMN functions in the same pathway as CUBN. We demonstrate that Cubn is not properly localized to the cell surface in Ann−/− tissues in the embryo and adult mouse, and that adult chimeras exhibit selective proteinuria of Cubn ligands. This study demonstrates that Ann is an essential component of the Cubn receptor complex in vivo and suggests that Ann/Cubn is required for endocytosis/transcytosis of one or more ligands in the VE during gastrulation to coordinate growth and patterning of the embryo. Furthermore, as AMN is apparently not required for gastrulation in humans, the developmental requirements for Ann/Cubn function may not be evolutionarily conserved, possibly reflecting differences between species in the role and organization of extra-embryonic tissues.

Key words: Amnionless, Visceral endoderm, Kidney proximal tubules, Cubulin, Gastrulation

Introduction

Reciprocal interactions between the epiblast and surrounding extra-embryonic tissues mediate essential patterning events in the mouse gastrula (Lu et al., 2001). One extra-embryonic tissue, the visceral endoderm (VE), is a polarized epithelial cell layer that does not contribute directly to the fetus. At embryonic day (E) 5.5-6.0, prior to gastrulation, the VE completely encases the epiblast, with a thin basal lamina separating their basal surfaces (Fig. 1) (Dunn and Hogan, 2001; Kalantry et al., 2001). The apical surface of the VE faces maternal tissues and contains numerous microvilli and an extensive vesicular system, which function to absorb and digest nutrients from the maternal environment. In addition, the VE provides signals required for proper organization of the body axes. In particular the anterior visceral endoderm (AVE) secretes factors to promote correct positioning of the primitive streak and anterior patterning of the future central nervous system (Beddington and Robertson, 1999; Martinez-Barbera and Beddington, 2001; Perea-Gomez et al., 2002). Much less is known about the role of the VE in patterning the posterior region of the embryo. However, explant culture assays indicate that the VE supplies signals directing the differentiation of hematopoietic and endothelial cells from mesoderm emanating from the posteriorly located primitive streak (Belaoussoff et al., 1998).

Our earlier studies on the amnionless (ann) mutant highlighted a previously undefined role for the VE in patterning the primitive streak during gastrulation (Kalantry et al., 2001; Tomihara-Newberger et al., 1998). The primitive streak, a transient structure in amniotes, orchestrates epiblast cell behaviors required to generate definitive ectoderm, mesoderm and endoderm. Fate-mapping studies in the mouse
divide the primitive streak into three functional regions (Lawson et al., 1991). Proximal streak produces extra-embryonic mesoderm and germ cells; middle streak produces lateral plate, intermediate and paraxial components of trunk mesoderm; and distal streak produces cardiac mesoderm and node-derived axial mesendoderm. *amn* mutants do not assemble a functional middle streak and specifically fail to form non-midline trunk mesoderm (Tomihara-Newberger et al., 1998). In addition, *amn* mutants are growth impaired after E7.0, indicating that streak assembly is mechanistically tied to the growth of the embryo.

*Amn* encodes a novel predicted type I transmembrane protein of 458 amino acids (Kalantry et al., 2001). The only homology domain identified by sequence analysis is a stretch of 70 amino acids, displaying similarity to cysteine-rich (CR) regions present in a small group of proteins known to function as BMP inhibitors: chordin, short gastrulation and procollagen II A (Kalantry et al., 2001). During gastrulation, Amn is expressed exclusively on the apical surface of the VE facing the maternal environment (Kalantry et al., 2001). As the primary morphological defects of *amn* reside in epiblast derivatives, Amn acts cell non-autonomously in the VE to support epiblast cell behaviors required for assembly of a functional middle streak and growth of the embryo. It is unknown how Amn mediates these events and whether its function requires the CR region; however, its apical localization on the VE supports the hypothesis that Amn transports signaling molecules and/or nutritive factors from the maternal environment to the underlying epiblast.

We report here that mouse Amn is also expressed in kidney proximal tubules (KPT) and small intestine, two resorptive tissues (Kalantry et al., 2001). As the primary morphological defects of *amn* reside in epiblast derivatives, Amn acts cell non-autonomously in the VE to support epiblast cell behaviors required for assembly of a functional middle streak and growth of the embryo. It is unknown how Amn mediates these events and whether its function requires the CR region; however, its apical localization on the VE supports the hypothesis that Amn transports signaling molecules and/or nutritive factors from the maternal environment to the underlying epiblast.

While our analyses of *Amn*+/+/+ chimeras were in progress, Tanner et al. documented mutations in human *AMN* in five families with recessive hereditary megaloblastic anemia (MGA1, OMIM#261100; also known as Imerslund-Gräsbeck disease) (Tanner et al., 2003). MGA1, which is characterized by abnormally large erythroid precursors/erythrocytes and impaired DNA synthesis, results from a deficiency in vitamin B12 (Broch et al., 1984). Previously, 17 families with MGA1 were identified with mutations in cubulin (*CUBN*), which encodes the intrinsic factor (IF)-vitamin B12 receptor in the small intestine (Aminoff et al., 1999). CUBN, a 460 kDa glycoprotein, binds to numerous ligands, including IF-vitamin B12, apolipoprotein A1 (Apoa1), high density lipoprotein (HDL), transferrin and albumin (Muller et al., 2003). As CUBN lacks a transmembrane domain, it has been proposed that cubilin, a low-density lipoprotein receptor family member, stabilizes CUBN at the cell surface and mediates endocytosis of CUBN-bound ligands (Hammad et al., 2000; Moestrup et al., 1998). The association of both *AMN* and *CUBN* mutations with MGA1 argues that AMN, like CUBN, is required for vitamin B12 absorption and suggests that AMN function is intimately linked with CUBN function and possibly also megalin.

Exploring the relationship between Amn and Cubn, we have determined that Cubn is not appropriately localized to the apical cell surface of Amn-deficient VE and KPT. In addition, adult *Amn*+/+/+ chimeras excrete increased levels of the Cubn ligands albumin and transferrin in the urine. These data indicate that Amn is required for proper localization and function of Cubn in vivo. In addition, they support a model in which endocytosis/transcytosis by Amn/Cubn on the VE provides a factor(s) to the epiblast essential for primitive streak assembly and function during gastrulation. The *amn* mutant phenotype highlights the role of extra-embryonic tissues in the coordination of growth and patterning in the gastrulation-stage mouse embryo. While Amn is required for murine gastrulation, it is apparently not essential for human gastrulation (Tanner et al., 2003), a finding that probably reflects distinct anatomical differences in the organization of extra-embryonic tissues relative to the epiblast, as well as different mechanisms for nutrient and waste exchange between mouse and human embryos.

**Materials and methods**

**In situ hybridization and immunohistochemistry**

In situ hybridization studies were carried out as previously described (Kalantry et al., 2001; Tomihara-Newberger et al., 1998). Immunohistochemistry was performed with the Discovery staining module at the MSKCC Molecular Cytology Core Facility (Ventana Medical Systems). Dilutions used were Biotinylated Lotus Tetragonolobus Lectin (LTL) (Vector Laboratories) at 20 μg/ml, goat anti-human megalin (Santa Cruz Biotechnology) at 1:800, goat anti-human CUBN (Santa Cruz Biotechnology) at 1:500, rabbit anti-human lysozyme (Dako) at 1:1000, and rabbit anti-mouse Amn as previously described (Kalantry et al., 2001). The analysis was performed with a Zeiss Axioplan microscope. Immunofluorescent antibody staining was performed on cryosections of fresh frozen intestine with rabbit anti-mouse Amn (1:50) and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) and analyzed by confocal microscopy (Zeiss LSM-510).

**Generation of *Amn*+/++ blastocyst chimeras**

ESC were derived from blastocysts generated by intercrosses of 129Sv *Amn*+/− mice or 129/B6 *Amn*−/− mice, as described (Nagy et al., 2003). From 200 blastocysts, 39 ESC lines were established and genotyped by PCR as previously described (Kalantry et al., 2001; Wang et al., 1996). We identified two *Amn* mutant ESC lines, line 9 (*Amn*+/−/−) and line 2–41 (*Amn*−/−/−), which are interchangeably
designated as Amn<sup>–/–</sup> ESC. Blastocysts from B6×B6/129 ROSA26 crosses were injected with Amn<sup>+/–</sup> ESC or Amn<sup>+/+</sup> ESC, surgically transferred into the uteri of pseudopregnant foster mothers, and allowed to develop to E16.5 or to term. Wild-type cells were transferred into the uteri of pseudopregnant foster mothers, and distinguished by β-galactosidase activity as described (Nagy et al., 2003). For live-born chimeras, contribution of Amn<sup>+/–</sup> cells was estimated by X-gal staining of tail tips. In adult chimeric tissues, Amn-deficient tissues were identified by the absence of Amn antibody staining.

**Urinalysis and Western blotting analysis**

Overnight collection of urine samples from adult chimeras was performed in metabolic cages (Lab Products). In the urine samples, creatinine and albumin were measured respectively by the Creatinine Companion kit and the Albuwell M kit (Exocell). Transferrin was measured by the Mouse Serum Transferrin kit (Alpha Diagnostic) with modifications suggested by the company for urine samples. For western blotting analysis, loading volumes were normalized by creatinine values. They were equivalent to 163 ng creatinine (albumin) or 2.45 µg creatinine (transferrin and lysozyme).

Western blotting analysis was performed according to standard protocols using ECL Plus Western blotting detection reagents (Amersham Biosciences). Antibody dilutions used were goat anti-mouse albumin (Bethyl Laboratories) at 1:10,000, rabbit anti-human transferrin (Dako) at 1:1000 and rabbit anti-human lysozyme (Dako) at 1:1000.

Western blotting analysis was performed using ECL Plus Western blotting detection reagents (Amersham Biosciences). Antibody dilutions used were goat anti-mouse albumin (Bethyl Laboratories) at 1:10,000, rabbit anti-human transferrin (Dako) at 1:1000 and rabbit anti-human lysozyme (Dako) at 1:1000.

**Results**

**Amn is expressed in kidney proximal tubules and intestinal epithelium**

During fetal development in the mouse, Amn transcripts are detected in kidney and intestine (Fig. 2A). Amn is expressed in mesonephric tubules at E11.5-12.5 and in the metanephric kidney beginning at E14.5 (data not shown). Amn expression, maintained in adult kidney, is observed in the outer cortex and the outer stripe of the outer medulla, but not in the inner medulla (Fig. 2B). The cortical expression of Amn is associated specifically with the epithelia of the small intestine (Fig. 2D-E), with the protein predominantly localized to the apical surface (Fig. 2F).

**Amn is not required for proliferation or differentiation in fetal kidney or intestine**

To determine whether Amn plays a cell-autonomous or cell non-autonomous role in organogenesis, chimeras were constructed by injection of Amn<sup>–/–</sup> ESC into ROSA26+/+ blastocysts (Amn<sup>+/+</sup> homozygous wild-type, Amn<sup>–/–</sup> homozygous mutant, Amn<sup>+/–</sup> heterozygous) and ROSA26 mice constitutively express β-galactosidase in nearly all cell types; consequently +/+ cells are distinguished from Amn<sup>–/–</sup> cells by X-gal staining (Zambrowicz et al., 1997).

If Amn is required for cell proliferation in kidney and intestine, Amn<sup>–/–</sup> cells will be underrepresented when compared to +/+ cells in these fetal chimeric tissues. As shown in Fig. 3, Amn<sup>–/–</sup> cells (red arrows) efficiently contribute to both fetal kidney tubules (Fig. 3A) and intestinal epithelium (Fig. 3B). Furthermore, we observed from zero to nearly 100% contribution of Amn<sup>–/–</sup> cells in both Amn-expressing (kidney and intestine) and non-expressing (liver) tissues in individual animals. Similar results were obtained in control Amn<sup>+/+</sup> chimeras (data not shown). Thus, Amn is not required for cell proliferation in these tissues during fetal development.

In chimeric embryos containing nearly 100% Amn<sup>–/–</sup> cells, the kidneys and intestine displayed an overtly normal morphology. Amn-deficient kidneys were of appropriate size and contained readily identifiable glomerular and tubular structures (Fig. 3C). Similarly, Amn-deficient intestine contained well-formed, apically located villi (Fig. 3D).

To assess whether Amn functions in cell differentiation, we examined the lectin-staining profile of Amn-deficient KPT. Amn-deficient kidneys were stained with LTL, which specifically recognizes KPT (D’Agati and Trudel, 1992). As shown in Fig. 3F, the complete absence of X-gal staining
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Fig. 3. Amn is not required for cell proliferation or differentiation in fetal kidney and intestine. Cryosections from E16.5 Amn\(^{-/-}\)/+/+ chimeras. (A,C) Two kidney sections and (B,D) two intestine sections stained with X-gal to identify wild-type (blue cells indicated by blue arrows) and Amn\(^{-/-}\) cells (pink cells indicated by red arrows). (A) Amn\(^{-/-}\) cells colonize the kidney and contribute to glomeruli (G) and tubules. (B) Amn\(^{-/-}\) cells colonize the intestinal villi (V). (C) Section of a chimeric kidney containing ~100% Amn\(^{-/-}\) cells with normal gross morphology, including properly formed glomeruli (G) and tubules. (D) A section of chimeric intestine containing ~100% Amn\(^{-/-}\) cells with normal morphology and well-formed villi (V). (E) Chimeric kidney section stained with LTL. (Inset) Kidney cryosection from an E16.5 Amn\(^{-/-}\)/+/+ chimera stained with LTL. (F) X-gal staining of section adjacent to (E) identifies Amn\(^{-/-}\) cells in properly differentiated proximal tubules (PT). (Inset) X-gal staining of kidney cryosection from an E16.5 Amn\(^{-/-}\)/+/+ chimera.

identified tubules composed of 100% Amn\(^{-/-}\) cells; such tubules labeled robustly with LTL (Fig. 3E). Therefore, Amn is not required cell-autonomously for differentiation of KPT cells or cell non-autonomously for the development of any other component of the nephron.

Cubilin is not properly localized to the apical cell surface of Amn-deficient cells

The finding that mutations in either CUBN or AMN independently cause human MGA1 argues that the two proteins act in the same endocytic pathway. A canine model for MGA1 provides a further clue to the nature of this interaction. While affected dogs do not bear mutations in Cubn, Cubn itself is mislocalized, suggesting that another protein is required to insert Cubn on the apical membrane (Fyfe et al., 1991a; Fyfe et al., 1991b). As shown in Fig. 4A-C, Amn, Cubn and megalin co-localize to the apical surface of the VE in E7.5 wild-type mouse embryos. Such co-localization supports a possible functional relationship among Amn, Cubn and megalin in the mouse. In amn, megalin is appropriately localized to the apical surface of the VE (Fig. 4D,d). Furthermore, antibody staining indicates that Amn-deficient VE takes up lysozyme, a megalin-specific ligand (Orlando et al., 1998), revealing that megalin is functionally intact (insets in Fig. 4a,d). By contrast, Cubn fails to localize to the apical surface of the VE in amn (Fig. 4f). Additionally, as mislocalization of Cubn is observed at E6.5 prior to the morphological appearance of the amn phenotype, it is not a secondary consequence of the amn phenotype (data not shown). Interestingly, Cubn is mislocalized, despite proper megalin expression and function on Amn-deficient VE. Thus, contrary to previously proposed models, megalin is not sufficient while Amn is essential for Cubn localization in the VE.

We have also examined the localization of Cubn and megalin in KPT of an adult chimera with ~70% contribution of Amn\(^{-/-}\) cells in the kidney. The chimeric kidney is internally controlled as it contains both Amn\(^{+/+}\) wild-type KPT (Fig. 5A,D,G, blue asterisks), identified by apical staining with Amn antisera (Fig. 5F; WT), and Amn\(^{-/-}\) mutant KPT (Fig. 5A,D,G, red asterisks), recognized by the lack of Amn expression but presence of megalin expression. In wild-type KPT, Amn, Cubn and megalin co-localize to the apical surface (Fig. 5C,F,I; WT). In Amn\(^{-/-}\) KPT, megalin is properly localized (Fig. 5G, red asterisk; 5H, mut). Significantly and similar to Amn-deficient VE, Cubn is not detected on the apical surface of Amn-deficient KPT but is detected in a punctuate pattern in the cytoplasm of KPT cells (Fig. 5A, red asterisk; 5B, mut). Therefore, Amn is required for apical expression of Cubn in adult tissues as well as in the VE.

Adult chimeras with high contribution of Amn\(^{-/-}\) cells excrete increased levels of Cubn-specific ligands in urine

To assess the role of Amn in kidney function, urine samples of adult chimeras were normalized by creatinine and analyzed by SDS-PAGE and western blotting. As creatinine is freely filtered by the glomerulus and not metabolized by the kidney, it is routinely used as an internal standard against which the levels of other metabolized urinary proteins are measured (Anker, 1954; Blumenfeld and Vaughan, 2002). The analysis included six males and seven females with estimated contribution of Amn\(^{-/-}\) cells ranging from 0-70%. When compared with C57BL/6J and 129/SvImJ control animals, the urine of chimeras with high contribution of Amn\(^{-/-}\) cells contained visibly elevated levels of one protein, which was identified as albumin by western blotting analysis (Fig. 6A,B). Protein levels in the urine of normal mice vary significantly between mouse strains and between males and females, with males generally having higher protein levels (Finlayson and Baumann, 1958). Thus, with only one high contribution male chimera, we used only the female chimeras for quantitation. The albumin/creatinine excretion ratio ranged from 2.6-295.2 µg albumin/mg creatinine in female chimeras (seven animals), whereas control females ranged from 5.4-26.5 µg albumin/mg creatinine (three animals) (Fig. 6C).

Albumin binds both Cubn and megalin; consequently, albuminuria does not distinguish between functional defects in Cubn and megalin. Therefore, we also monitored the concentration of a Cubn-specific ligand, transferrin, and of a megalin-specific ligand, lysozyme, in urine. Increased levels of transferrin were detected only in high contribution chimeras (Fig. 6A,B). The transferrin/creatinine excretion ratio ranged
from 0-1.5 ng transferrin/mg creatinine in female chimeras (seven animals) and from 0-0.6 ng transferrin/mg creatinine in female controls (three animals) (Fig. 6C). The megalin-specific ligand, lysozyme, was found in similar quantities in the urine of male chimeras and controls, but was variable in urine samples of female chimeras and controls, most probably because of the mixed genetic background of the chimeras (Fig. 6A,B). In particular, 129 females have no detectable lysozyme while lysozyme is detectable in the urine of B6 females. Similarly, chimeras with high contribution from 129 homozygous ES cells do not excrete detectable levels of lysozyme. The range of lysozyme excretion observed in high contribution chimeras is similar to that observed in control animals (Fig. 6A). The excretion of DBP, another megalin-specific ligand, was also variable; however, the range of DBP excretion did not differ from that observed in control animals (data not shown). Thus, Amn appears to be crucial for Cubn function in the adult kidney while megalin function is intact.

Discussion

Amn is required for Cubn expression on the apical surface of polarized epithelial cell types

We have identified only three cell types that express Amn in the mouse; each is a polarized epithelial cell specialized for resorption and/or transport: the VE of the embryo, the KPT and the epithelium of the small intestine. Amn probably functions in a process common to all three cell types. An important clue to Amn function has been revealed by genetic analyses of families with MGA1, an inherited human autosomal recessive disease characterized by intestinal vitamin B12 malabsorption. These families carry mutations in either CUBN, the biochemically identified IF-vitamin B12 receptor (Seetharam et al., 1981; Seetharam et al., 1997), or in AMN. This finding argues that AMN and CUBN are functionally related, as both are required for vitamin B12 uptake by the small intestine.

Fig. 4. Cubn is not properly localized to the apical cell surface of the VE in amn. Immunohistochemical analyses of serial paraffin sections of an E7.5 wild-type embryo (A-C) and an E7.5 amn mutant (D-F), which were counterstained with Hematoxylin. The regions designated by brackets in A-F are shown at high magnification in a-f. (A,a) Megalin, (B,b) Amn and (C,c) Cubn co-localize to the apical cell surface (red asterisks) of the VE in the wild-type embryos. The basal cell surface faces the epiblast (red arrowheads). (D,d) Megalin staining of the apical cell surface of the VE in amn, identified by the absence of Amn antibody staining in the adjacent section (E,e). (F,f) Cubn appears cytoplasmic and not apical in amn. (a and d, inset) Lysozyme staining in wild-type (a) and amn mutant (d) embryos.

Fig. 5. Cubn is not localized to the apical cell surface in Amn-deficient KPT. Immunohistochemical analyses of serial paraffin sections (A,D,G) from an adult chimeric kidney with high contribution of Amn−/− cells. Two sets of serial high magnification views are shown in B,E,H and C,F,I. (A-C) Cubn, (D-F) Amn and (G-I) megalin antibody staining of wild-type KPT (blue asterisks; WT) and Amn-deficient KPT (red asterisks; mut). A chimeric tubule (green asterisks; ch), which contains both wild-type and Amn−/− cells, is designated as well as a distal tubule (yellow asterisks; DT). Of significance, Cubn does not localize to the apical surface in Amn-deficient KPT but is observed in the cytoplasm (B,C).
As predicted by their proposed interdependent function, Amn and Cubn are co-expressed in small intestine and in KPT in mouse, human and dog, as well as in the mouse VE. AMN and CUBN are both required in humans, and most probably dogs, for IF-vitamin B12 absorption by the intestine. Amn-deficient intestine in the mouse appears to develop properly and analysis of blood samples of high contribution chimeras did not show evidence of megaloblastic anemia (data not shown). Indeed, as adult Amn+/++/+ cells, a small number of Amn-expressing intestinal cells may supply the animal with sufficient supplies of vitamin B12. Conditional knockout strategies are in progress to assess whether mice with Amn-deficient intestines display symptoms of megaloblastic anemia.

Proteinuria, which is often associated with MGA1, probably reflects a deficiency in an endocytic function of AMN/CUBN within KPT. Likewise, dogs with mislocalized Cubn excrete approximately seven times more albumin in urine than do wild-type controls (Birn et al., 2000). The Amn+/++/+ chimeras described in this study also present with defects in KPT function. In particular, selective proteinuria is observed for Cubn-bound ligands, such as albumin and transferrin. However, megalin-bound ligands appear unaffected, pointing to Amn/Cubn-specific, megalin-independent, endocytic functions in the KPT. In agreement with Amn/Cubn acting as a separate endocytic receptor complex, Fyfe et al. have found that following transfection of AMN and CUBN expression vectors, CHO cells were able to endocytose IF-vitamin B12 (Fyfe et al., 2004).

**Potential ligands for the Amn/Cubn complex in the mouse gastrula**

Although the crucial ligand for AMN/CUBN in the intestine is IF-vitamin B12, the crucial ligand during murine gastrulation has not been identified. IF-vitamin B12 is not a likely candidate, as it is found only in the stomach/small intestine. Cubn, a scavenger receptor, binds to numerous ligands; thus, Amn/Cubn may be required during mouse gastrulation for the uptake of one or more of these from the maternal circulation. Known Cubn-specific ligands, such as Apoa1/HDL and transferrin, are candidates for Amn/Cubn transport (Hammad et al., 1999; Kozyraki et al., 1999). However, Apoa1/HDL are not strong candidates for the crucial Amn/Cubn ligand during murine gastrulation, as Apoa1-deficient mice are viable and fertile (Li et al., 1993; Williamson et al., 1992). Transferrin, however, cannot as yet be ruled out as a crucial Amn/Cubn ligand in the mouse embryo. As most plasma iron circulates bound to transferrin, Cubn-mediated uptake of transferrin may release iron and provide it to the embryo. However, there are other routes for cellular uptake of iron, including binding of transferrin to the transferrin receptor (Trfr) and Fe3+ transport by the transmembrane iron transporter DMT1 (Andrews et al., 2000). It is not known whether DMT1 is active on the apical surface of the VE, but Trfr is expressed by the mouse yolk sac and a Trfr deficiency results in lethality by E12.5, with defective erythropoiesis and neurological development (Andrews et al., 2000). Trfr-deficient embryos show signs of anemia only after E10.5, suggesting the existence of an alternative pathway of iron uptake prior to E10.5, perhaps Amn/Cubn. Alternatively, it is likely that other, presently unknown, Cubn-specific ligands are endocytosed/tra...
Developmental requirements for Amn/Cubn function differ between rodents and human/dog

Although the requirement for Amn/Cubn function in intestine and KPT appears conserved across mammalian species, the same cannot be said for the role of Amn during embryonic development. Amn is required during murine gastrulation for survival; yet humans and dogs have no apparent need for Amn/CUBN until after birth. While a *Cubn*-null mutation has not yet been reported in mouse, antibodies to Cubn, but not to megalin, induce fetal malformations in rats (Brent and Fawcett, 1998; Sahali et al., 1988; Seetharam et al., 1997). If Amn solely functions in a complex with Cubn, *Cubn*-null mutations in the mouse will result in a gastrulation phenotype identical to *amn*.

The differential requirement for Amn/Cubn during embryogenesis in rodents versus humans/dogs may reflect anatomical differences in the organization of extra-embryonic and embryonic tissues, as well as in mechanisms for nutrient and waste exchange. The murine visceral yolk sac, formed by an outer VE layer and an inner extra-embryonic mesoderm layer at ~E7.5, completely surrounds the developing fetus. It serves as a maternal-fetal interface required for the exchange of nutrients, oxygen and waste products (Rossant and Cross, 2002). By contrast, the human yolk sac becomes a vestigial appendage attached near the allantoic mesoderm (Sadler, 1995), and trophoblast derivatives act as the major maternal-fetal interface supplying required nutrients and factors. The differences in the role of the yolk sac between species is exemplified by mutations in ApoB that cause embryonic lethality in the mouse but fail to affect normal human development (Farese et al., 1995; Hopkins et al., 1986; Shi and Heath, 1984).

Does the *amn* mutant phenotype result solely from a general nutritional deficiency or does it reflect both the absence of a nutritional factor and a signaling or patterning molecule?

The finding that 4793 Amn is a component of an endocytic scavenger receptor raises the question of whether the distinct features of the *amn* mutant phenotype result from a nutritional deficiency that impairs the general growth of the embryo. Although *amn* mutants are small, not all tissues are similarly compromised. The anterior (head, heart, and node derivatives) and extra-embryonic regions are appropriately specified; yet the middle streak fails to assemble and trunk mesoderm is never produced (Tomihara-Newberger et al., 1998). Thus, this phenotype is unlikely to result simply from an overall delay in growth.

The *amn* phenotype is very distinct from those found in other mutants with defects in VE transport/trafficking functions. Ubiquitously expressed Sec8 is a member of the Sec6/8 complex, which regulates delivery of exocytic vesicles to plasma membrane docking sites (Friedrich et al., 1997). At E7.5, *sec8* mutant embryos are developmentally delayed by ~24 hours, but they still express *Brachyury* (*T*), a marker of nascent mesoderm, but not *Mox1*, a marker for paraxial mesoderm (Friedrich et al., 1997), suggesting either delayed marker expression or simply arrest during gastrulation (Yeaman et al., 2001). A very similar early gastrulation-stage phenotype is observed in *Hnf4*+/− embryos. Hnf4, which is expressed exclusively in VE during gastrulation stages, is a positive transcriptional activator of genes required for secretion, transcytosis, and digestion (Duncan et al., 1994; Duncan et al., 1997). *Hnf4* is not required for early specification of VE but is required for its complete differentiation, as *Hnf4*-deficient VE fails to express various VE markers (Duncan et al., 1997).

Knockout mutations in mouse *Hβ58* (Vps26 – Mouse Genome Informatics), *Snx1* and *Snx2* provide further evidence that transport/trafficking functions are required for midgestation development. *Hβ58*, expressed at low levels in the epiblast and at high levels in the VE, is the homolog of yeast Vps26p, a member of the retromer complex that mediates endosome-to-Golgi trafficking (Lee et al., 1992; Seaman et al., 1998). The sorting nexins 1 (*Snx1*) and 2 (*Snx2*) are homologs of yeast Vps5p, also a component of the retromer complex (Seaman et al., 1998). *Snx1*+/−; *Snx2*+/− double mutants display a phenotype nearly identical to that of *Hβ58* mutants, which are visibly growth retarded at ~E7.5 and developmentally arrested by E10.5 (Schwarz et al., 2002). Thus, *Hβ58* and *Snx1* and *Snx2* appear to function in the same cellular trafficking pathway, which probably acts to maintain functional integrity of the VE. Notably, despite their severely retarded growth, and in contrast to *amn* mutants, *Hβ58* and *Snx1*+/−; *Snx2*+/− mutants form derivatives of all three germ layers, including somites (Radice et al., 1991; Schwarz et al., 2002).

The *sec8* and *hnf4* mutant phenotypes are more severe than that of *amn*, including growth retardation and failure to support gastrulation. The *Hβ58* and *Snx1*+/−; *Snx2*+/− mutant phenotypes, however, are less severe. The mutant embryos are very small but undergo gastrulation and produce trunk mesoderm and somites. Although it has not been determined whether the defects in these mutants are solely the result of blocked endocytosis/transcytosis, the comparison of these mutant phenotypes suggests that the middle streak defects in *amn* do not simply result from a general delay in embryonic growth. Thus, loss of Amn not only impairs growth of the epiblast, but also disrupts mechanisms for assembling an appropriately patterned and functional middle primitive streak. A question for future studies is how, via endocytosis, transcytosis, and/or signaling, Amn/Cubn promotes and coordinates growth and patterning of the mouse gastrula.

In summary, Amn mediates Cubn localization and function in the mouse, which argues for an essential role of Amn/Cubn-directed endocytosis/transcytosis in the VE during gastrulation. We are currently considering two general models to explain the combined defects in growth and middle primitive streak assembly/function in the *amn* mutant. In the first model, Amn/Cubn-mediated endocytosis/transcytosis in the VE may provide nutrient(s) to the epiblast that are essential for cell proliferation and growth. However, loss of Amn would not equally compromise all proliferating epiblast cells; those that assemble into the middle streak, thereby physically separating distal and proximal streak regions, would be the most severely affected. In the second model, Amn/Cubn-mediated endocytosis/transcytosis in the VE, in addition to providing essential nutrients required for general growth, would facilitate/modulate key signaling pathways required for specification of the middle streak and its derivatives. Further dissection of the *amn* mutant phenotype and the nature of the
requirement for Amn during gastrulation will provide insight into the role of the VE in murine development and highlight similarities and differences in gastrulation between species.

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References


Perea-Gomez, A., Vella, F. D., Shawlot, W., Oulad-Abdelghani, M.,
Amn mediates Cubn function in the VE and KPT


