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CELLULAR UPTAKE OF STEROID CARRIER PROTEINS – MECHANISMS AND IMPLICATIONS

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ABSTRACT

Steroid hormones are believed to enter cells solely by free diffusion through the plasma membrane. However, recent studies suggest the existence of cellular uptake pathways for carrier-bound steroids. Similar to the clearance of cholesterol via lipoproteins, these pathways involve the recognition of carrier proteins by endocytic receptors on the surface of target cells, followed by internalization and cellular delivery of the bound sterols. Here, we discuss the emerging concept that steroid hormones can selectively enter steroidogenic tissues by receptor-mediated endocytosis; and we discuss the implications of these uptake pathways for steroid hormone metabolism and action *in vivo*.

Introduction: Endocytosis mediates cellular uptake of cholesterol

All sterols transported in plasma or extracellular fluids are solubilized by proteins. When contemplating mechanisms how to deliver circulating sterols to cells, it may be quite insightful to consider how this task is achieved for the prototype sterol cholesterol.

Transport of cholesterol in the extracellular space is facilitated by a special protein complex called lipoprotein (Havel and Kane, 2001). Lipoproteins are spherical macromolecules of 10 to 1200 nm diameter composed of a core of neutral lipids (mostly cholesterol ester and triglycerides) surrounded by an amphipathic shell of polar phospholipids and cholesterol. Embedded in the shell of lipoproteins are apoproteins that are essential for assembly of the particles and for their recognition by cells (Havel and Kane, 2001). Lipoproteins traffic cholesterol from the tissue of origin to target sites where the lipid cargo is delivered through receptor-mediated endocytosis (Fig. 1) (Goldstein et al., 2001). Delivery involves lipoprotein receptors on the surface of the cells that bind the apoprotein. Following interaction at the cell surface, receptor-ligand complexes are internalized and delivered to endosomal compartments. There, the receptors discharge their cargo. Un-liganded receptors recycle back to the cell surface while lipoproteins move to lysosomes where they are catabolized. The apoprotein moiety is degraded into small peptides and the lipids are released (Goldstein et al., 2001). Cholesterol enters the cellular membrane pool via the Endoplasmatic Reticulum, is converted into steroid hormones in mitochondria, or stored as cholesterol esters in cytoplasmic lipid droplets (Fig. 1). The exact mechanism of cholesterol trafficking between cellular compartments is still a matter of investigation (Prinz, 2002). Trafficking critically depends on the activity of intracellular sterol carrier proteins that solubilize the sterol and direct its transport

(Liu, 2009). For example, exit of cholesterol from lysosomes requires the activity of two proteins designated Niemann-Pick disease type C protein 1 (NPC-1) and 2 (NPC-2). NPC-1 is a polytopic membrane protein with sterol-sensing domain that acts as cholesterol transporter across membranes (Kwon et al., 2009; Zhang et al., 2001). NPC-2 is a small cholesterol binding protein possibly involved in shuttling sterols between membranes (Naureckiene et al., 2000; Okamura et al., 1999).

The composition of apoproteins provides a unique signature of individual lipoprotein classes specifying the origin and types of lipids transported, and their destiny. Target cells express a unique set of lipoprotein receptors on their surface that are able to discriminate various lipoprotein species by recognition of their specific apoprotein profile. Thus, receptor-mediated endocytosis provides an efficient and highly selective mechanism for directing cholesterol and other lipids into their proper target tissue.

The main class of lipoprotein receptors is a group of cell surface proteins called the low-density lipoprotein (LDL) receptor gene family (Fig. 2) (Beffert et al., 2004; Herz and Hui, 2004; Schneider, 2007; Willnow et al., 1999). Family members are expressed in many tissues in organisms as distantly related as nematodes and mammals. The LDL receptor is the archetype of the gene family and has a structure and function typical of a receptor involved in cellular cholesterol uptake (Fig. 2). Its significance for systemic cholesterol homeostasis is underscored by pathological features in patients with Familial Hypercholesterolemia, inheritable LDL receptor gene defects that result in an inability of affected individuals to clear cholesterol-rich lipoproteins from the circulation (Goldstein et al., 2001). Other members of the gene family with confirmed roles in lipoprotein metabolism include the LDL receptor-related protein (LRP) 1, a receptor for clearance of dietary lipids in the liver

(Rohlmann et al., 1998). Very-low density lipoprotein (VLDL) receptors and yolkless are expressed in oocytes of egg-laying species from insects to birds and mediate the endocytic uptake of yolk (Bujo et al., 1995; Grant and Hirsh, 1999; Schonbaum et al., 1995).

Receptors for steroid carriers – surface binding sites for sterol signaling and uptake

In contrast to the precursor cholesterol, cholesterol-derived steroid hormones are not transported by lipoproteins but by plasma carrier proteins. Carrier proteins are unique for individual classes of steroid hormones. They include the vitamin D binding protein (DBP; the carrier for vitamin D metabolites) (White and Cooke, 2000), the sex hormone-binding globulin (SHBG; the carrier for androgens and estrogens) (Hammond and Bocchinfuso, 1995), and the corticosteroid-binding globulin (CBG; the carrier for glucocorticoids) (Scrocchi et al., 1993). Curiously, steroid hormones bound by carrier proteins are considered biologically inactive. Instead it is the free steroid that is proposed to enter target cells by unspecific diffusion through the plasma membrane following release from its carrier. In this model, binding of sterols to carriers provides a reservoir of inactive circulating steroids and it regulates the amount of free hormones available for diffusion into tissues. This model is known as the free hormone hypothesis (Mendel, 1989).

Ample evidence from studies in humans and experimental models supports the free hormone hypothesis. However, distinct expression of carriers in steroid-target tissues also suggests roles for these proteins in local steroid hormone action (Hryb et al., 2002; Kahn et al., 2002). Over the years there have been consistent reports

describing the existence of surface binding sites for steroid hormone carriers on tissues, suggesting alternative pathways for targeting protein-bound sterols to distinct cell types. Initial reports have mainly used biochemical approaches to detect interaction sites for steroid carriers in membrane preparations. Among other tissues, binding sites for SHBG have been documented in prostate, testis, epididymis and endometrium (Feldman et al., 1981; Fortunati et al., 1991; Frairia et al., 1991; Hilpert et al., 2001; Hryb et al., 1985; Krupenko et al., 1994; Porto et al., 1995), and in established cell lines such as the estrogen-dependent breast cancer cell line MCF-7 (Catalano et al., 2005; Fortunati et al., 1998; Nakhla et al., 1999; Rosner et al., 1999). Surface binding sites for CBG have been shown in liver, endometrium, placenta, prostate, spleen, and kidney (Hryb et al., 1986; Maitra et al., 1993; Singer et al., 1988; Strel'chyonok and Avvakumov, 1991). Potential receptors for DBP have been reported on macrophages, trophoblasts, and neutrophils (DiMartino et al., 2007; Gumireddy et al., 2003; Haddad, 1995).

Surface binding sites for steroid carriers are implicated in different cellular processes (Caldwell et al., 2006). They are proposed to act as receptors for transmembrane signaling of steroid hormones, a pathway that does not require steroids to enter cells or to interact with nuclear hormone receptors (non-genomic action). Most extensively, this activity (tentatively named R_{SHBG}) has been studied in the breast cancer cell line MCF7. In this cell type, SHBG has been implicated in cAMP-dependent signaling of bound-estradiol (Heinlein and Chang, 2002; Kahn et al., 2008; Nakhla et al., 1999; Rosner et al., 1999). Furthermore, the carrier has been shown to antagonize estradiol-induced proliferation of these cells by inhibiting the activation of extracellular regulated kinase (ERK) -1/-2 (Catalano et al., 2005) and by regulating expression of downstream target genes involved in cell growth and

apoptosis (Catalano et al., 2007; Fortunati and Catalano, 2006). These concepts are described in detail elsewhere in this issue. Stimulation of membrane receptors by SHBG and down-stream signaling through MAP kinase pathways has also been reported in neuronal cell lines (Caldwell et al., 2006).

In addition, surface binding sites for carrier proteins have been implicated in local sequestration of steroids, thereby enhancing the free diffusion of hormones into tissues (Janne et al., 1998). Notably, Hammond and coworkers identified fibulin-1D and fibulin-2, components of the extracellular matrix as binding sites for SHBG. Interaction of SHBG with the extracellular matrix is dependent on the presence of sex steroids and facilitates accumulation of SHBG-bound estrogens in the stroma of the endometrium (Ng et al., 2006).

Last but not least, surface binding sites have been proposed to mediate the cellular uptake of carrier-bound steroid hormones. For example, uptake of SHBG has been documented in epithelial cells of the epididymis and endometrium (Feldman et al., 1981; Gerard et al., 1988; Noe et al., 1992), in MCF-7 cells (Porto et al., 1991), and in neurons in the brain (Caldwell et al., 2007). A biological process, where uptake of SHBG is suggested to play a physiological role is the delivery of testosterone to principal cells in the epididymis (Fig. 3). In male rodents, SHBG (also known as androgen binding protein (ABP)) is produced by Sertoli cells in the testis and secreted into the lumen of the seminiferous tubules (Feldman et al., 1981; French and Ritzen, 1973). There it binds testosterone present in large amounts in the testicular fluids. Via the efferent ducts, SHBG is transported to the epididymis and internalized by principal cells lining the epididymal duct (Feldman et al., 1981; Gerard et al., 1988). Because principal cells are responsible for conversion of testosterone into dihydrotestosterone required for sperm maturation, endocytic uptake of SHBG is

proposed to provide a route for efficient delivery of testosterone to these cells (French and Ritzen, 1973; Gerard et al., 1988; Noe et al., 1992; Turner et al., 1995). The molecular identity of cell surface receptors for SHBG has not been revealed in previous reports. Binding studies indicated that this receptor activity binds SHBG with a K_d of 0.5 μM in solubilized prostate membranes (Hryb et al., 1989). By size chromatography an approximate molecular weight of 167 kDa was calculated (Hryb et al., 1989). Similarly, a SHBG receptor activity was also partially purified from human endometrium (Fortunati et al., 1992).

Megalin, a receptor for cellular uptake of vitamin D metabolites bound to DBP

The first receptor for uptake of carrier-bound steroids identified and characterized in detail is megalin, a member of the LDL receptor gene family (Fig. 2). Megalin (or LRP2) is 600 kDa protein specifically expressed on the apical surface of absorptive epithelia (Saito et al., 1994). Prominent sites of expression in the adult organism are the proximal tubules in the kidney, the uterine epithelium, the epididymis, the mammary gland, as well as the ependyma in the brain ventricles (Zheng et al., 1994). During embryonic development, highest expression of the receptor is seen in the visceral endoderm of the yolk sac and the epithelial cells of the neural tube, the progenitor of the developing central nervous system (Assemat et al., 2005). An appreciation of the significance of megalin for embryonic development came with the generation of mouse model carrying a targeted disruption of the murine *megalin* gene. Animals genetically deficient for the receptor suffer from malformation of the forebrain characterized by fusion of the forebrain hemispheres, lack of the corpus callosum, and incompletely developed eyes and olfactory bulbs (Willnow et al.,

1996). This syndrome is known as holoprosencephaly (HPE), a defect in formation of the embryonic mideline affecting as many as 1 in 250 pregnancies in humans (Wallis and Muenke, 1999). Intriguingly, several genes implicated in sterol metabolism cause HPE when mutated in patients or animal models . These genes encode 7-dehydrocholesterol reductase, an enzyme in the cholesterol biosynthetic pathway (Kelley, 2000; Kelley and Hennekam, 2000), as well as sonic hedgehog, a morphogen that is covalently modified by cholesterol (Schell-Apacik et al., 2003). Recently, the relevance of megalin activity for embryonic brain formation was substantiated by the identification of *Megalin* gene defects in patients suffering from Donnai-Barrow syndrome, an autosomal recessive disorder characterized by various brain anomalies (Kantarci et al., 2007). Whether or not the role of megalin in forebrain patterning involves a function in embryonic sterol metabolism is unclear as yet.

Previously, the functional elucidation of megalin had been hampered by the fact that its physiological ligands were unknown. *In vitro* and in cultured cells, a plethora of ligands had been indentified that bound to this receptor. Apart form apolipoprotein (apo) B100 and apoE, potential ligands included proteases and protease inhibitors, enzymes, and peptide hormones (reviewed in (Christensen and Birn, 2002; Willnow et al., 1999)). A clue as to the true ligands of this receptor came with the analysis of mice with ubiquitous or kidney-specific megalin gene defects (Leheste et al., 1999; Nykjaer et al., 1999). Based on the prominent expression of megalin on the luminal surface of the renal proximal tubules, a role for the receptor in retrieval of metabolites from the glomerular filtrate had been anticipated. Consistent with this assumption, mice lacking megalin expression exhibited tubular resorption deficiency and excreted a number of plasma protein normally reabsorbed by the receptor in the kidney. Intriguingly, ligands excreted by receptor-deficient animals

were low-molecular weight carrier proteins including DBP (Nykjaer et al., 1999), clara cell secretory protein (CCSP) (Burmeister et al., 2001), a carrier for progesterone, as well as transcobalamin and retinol binding protein, carriers for vitamin B12 and retinol, respectively (Birn et al., 2002; Christensen et al., 1999). Subsequent studies confirmed the relevance of megalin as a renal retrieval pathway for carrier-bound lipophilic vitamins and hormones essential to prevent uncontrolled urinary loss of these important metabolites (Christensen and Birn, 2001; Muller et al., 2003; Verroust et al., 2002).

Apart from elucidating a novel pathway in renal vitamin homeostasis, studies on the role of megalin in renal uptake of DBP arose considerable attention as they challenged the dogma stated by the free hormone hypothesis. 25-OH vitamin D_3 is the main vitamin D metabolite present in the circulation. It is an inert precursor that needs to be converted to 1,25-$(OH)_2$ vitamin D_3, the active hormone that regulates the systemic calcium homeostasis. Activation of 25-OH vitamin D_3 takes place in the proximal tubules of the kidney. In line with the free hormone hypothesis, 25-OH vitamin D_3 was proposed to enter the cells of the proximal tubule by free diffusion from the interstitial side. Given the large amounts of 1,25-$(OH)_2$ vitamin D_3 produced daily, dependence of the organism on unspecific diffusion processes to deliver the precursor sterol 25-OH vitamin D_3 selectively to this cell type in the body seemed awkward.

The 58 kDa DBP is the main plasma carrier for vitamin D metabolites (Cooke et al., 1991). It exhibits highest affinity for 25-OH vitamin D_3 (K_d 10^{-10} - 10^{-12} M) (Haddad, 1995; Haddad et al., 1981). Because DBP is present in 100-fold molar excess in the circulation, virtually all 25-OH vitamin D_3 is bound by this carrier (Bikle et al., 1986). Similar to other steroid carriers, DBP was believed to regulate the bioavailability of vitamin D metabolites and to protect the organism from excessive amounts of the free

sterol (Haddad, 1995). This concept had been challenged by findings obtained in DBP knockout mice. In these animals, lack of the carrier results in an inability to properly deliver 25-OH vitamin D_3 to the kidney for uptake and activation. Instead, most of the metabolite is wrongfully directed to the liver and catabolized, or lost in the kidney through urinary excretion (Safadi et al., 1999). A crucial role for the carrier in renal targeting and activation of 25-OH vitamin D_3 was also supported by the observation that DBP knockout mice are protected rather than sensitized to vitamin D toxification (Safadi et al., 1999).

Detailed analysis of mice genetically deficient for megalin uncovered the true mode of delivery of 25-OH vitamin D_3 to proximal tubule cells (Fig. 4). Contrary to previous hypotheses, it is not the free but the carrier-bound sterol that enters the cells. Uptake does not entail unspecific diffusion but relies on a highly specific uptake pathway involving an endocytic receptor for the carrier DBP. Delivery of the sterol does not proceed from the interstitium (basolateral side) but from the luminal (apical side) of the proximal tubule following glomerular filtration of the steroid/carrier complex. Absence of this DBP receptor pathway in *megalin*$^{-/-}$ mice results in an inability to retrieve 25-OH vitamin D_3/DBP complexes from the primary urine and in aberrant excretion of the metabolite. As a consequence of urinary loss of the steroid, plasma levels of 25-OH vitamin D_3 and 1,25-$(OH)_2$ vitamin D_3 are decreased by more than 70%, resulting in plasma vitamin D deficiency and in bone calcification defects (Hilpert et al., 2002; Leheste et al., 2003; Nykjaer et al., 1999).

A role for megalin in tubular clearance of DBP-bound vitamin D metabolites was confirmed in other models of impaired receptor function. In mice genetically deficient for the receptor-associated protein, a chaperone required for megalin biosynthesis (Willnow et al., 1996), the amount of megalin in the kidney is reduced by 50%. The reduction in

receptor expression results in urinary loss of DBP (Birn et al., 2000). Excretion of the DBP is also seen in rats treated with maleate, a substance that causes shedding of megalin from the luminal surface of the proximal tubules (Nagai et al., 2001). Patients with Donnai-Barrow syndrome where shown to excrete DBP as well (Kantarci et al., 2007). Finally, megalin-mediated uptake of DBP-bound vitamin D metabolites was substantiated in various cell lines such as osteoblasts, mammary epithelial cells, stellate cells, and in cells of the yolk sac (Chlon et al., 2008; Gressner et al., 2008; Nykjaer et al., 1999; Rowling et al., 2006).

In recent years, the molecular details of the endocytic machinery responsible for cellular uptake of DBP-bound sterols were unraveled. Nykjaer et al. identified cubilin, a second cell surface receptor for DBP in proximal tubule cells. Cubilin is a 460 kDa peripheral membrane protein that lacks transmembrane or cytoplasmic domains required for endocytosis. Thus, the receptor associates with megalin to recycle through the endocytic compartments of the cell (Burmeister et al., 2001; Hammad et al., 2000; Moestrup and Verroust, 2001). A role for cubilin in renal DBP uptake was uncovered, when DBP affinity chromatography was applied to identify membrane proteins in the kidney that may be involved in binding and cellular uptake of 25-OH vitamin D_3/DBP complexes. Besides megalin, the established DBP receptor, cubilin was purified (Nykjaer et al., 2001). Further analysis demonstrated that cubilin internalizes complexes of DBP and 25-OH vitamin D_3, and that cubilin deficiency in patients and in dogs with inheritable *cubilin* gene defects results in urinary loss of the steroid and in a decrease in plasma 25-OH vitamin D_3 and 1,25-$(OH)_2$ vitamin D_3 levels (Nykjaer et al., 2001). These findings suggested a two-receptor model for cellular uptake of the DBP/steroid complex (Fig. 4A).

Studies in patients and animal models with proximal tubular resorption deficiencies of various etiologies identified further components of the cellular machinery involved in delivery of the carrier DBP. Disabled (Dab) 2 is a cytoplasmic adaptor protein that binds to the cytoplasmic tail of megalin and that is required for proper routing of the receptor (Gotthardt et al., 2000). Inactivation of Dab2 in mice results in an inability of megalin to perform endocytosis. Consequently, Dab2-deficient mice are characterized by urinary loss of DBP (Morris et al., 2002). Like most proteins internalized by endocytic receptors, DBP is subject to lysosomal degradation, an efficient way to relieve 25-OH vitamin D_3 from its high affinity carrier. Proper function of the endocytic pathway in cells of the renal proximal tubule is dependent on the activity of the Cl^-/H^+ exchanger 5 (ClC-5) in endosomes (Plans et al., 2009). Patients suffering from inheritable ClC-5 gene defects (Dent´s disease) are characterized by disturbances in proximal tubular protein resorption and in calcium and bone metabolism (Lloyd et al., 1996; Pook et al., 1993). The underlying defect was identified as an inability of the tubule cells to perform megalin-mediated uptake of vitamin D metabolites bound to DBP (Plans et al., 2009). The same finding was obtained in mouse models of ClC-5 deficiency (Piwon et al., 2000; Wang et al., 2000).

Taken together, studies in humans and in animal models have firmly established the existence and the physiological importance of endocytic pathways for cellular uptake of the steroid carrier DBP in renal vitamin D homeostasis (Fig. 4B) (Negri, 2006; Willnow and Nykjaer, 2002).

Role of megalin in sex steroid action

Besides in the renal proximal tubules, megalin is also expressed in a number of other steroid-responsive tissues, in particular in the male and female reproductive organs (epididymis, ovaries, uterus) (Zheng et al., 1994). In the uterine epithelium, expression of the receptor is tightly regulated during estrus cycle, with strongest expression in met- und diestrus (Hammes et al., 2005). In fact, megalin has been identified as one of the major progesterone target genes in this tissue (Spencer et al., 1999). In addition, expression of the receptor has been detected in epithelial cells of the mammary gland and the prostate, tissues that give rise to sex steroid-dependent tumors (Chlon et al., 2008; Stanford et al., 1999). Collectively, these observations suggested the intriguing possibility that the role of megalin may not be restricted to endocytosis of vitamin D metabolites in the kidney, but extend to the cellular uptake of sex steroid in other organs.

Evidence for a physiological role of megalin in sex steroids action stems from the defects observed in the receptor-deficient mouse model. Apart from bone disease as a consequence of hypovitaminosis D (Nykjaer et al., 1999), adult *megalin*$^{-/-}$ animals suffer from anomalies in genital maturation consistent with insensitivity to androgens and estrogens. Despite normal (estradiol) or even increased levels (testosterone, DHT) of circulating hormones, these mice exhibit defects seen in rodents treated with anti-androgens and anti-estrogens. Thus, female receptor null mice fail to induce opening of the vagina cavity, a benchmark of natural puberty. This process can be induced in the immature rodent by injection of estradiol and blocked by application of anti-estrogens (Ashby et al., 2002). *Megalin*$^{-/-}$ males suffer from testicular maldescent. This process can be induced in offspring of pregnant rats and mice by treatment with flutamide, an inhibitor of the androgen receptor or with finasterite, a blocker of 5 alpha reductase that converts testosterone to DHT (Spencer et al., 1991).

Blockade of testicular descent in megalin knockkout mice was linked to a failure of affected males to induce regression of the cranial suspensory ligament (CSL), a tissue strand that tethers the cranial pole of the early embryonic gonad to the lateral body wall (Fig. 5B). In females, the CSL persists and holds the ovaries in a position close to the kidney. In males, the primordium of the CSL regresses during development enabling descent of the testes towards the lower abdomen (Fig. 5A). Involution of the CSL in males is androgen dependent. In mice lacking the androgen receptor (Hutson, 1986; Zimmermann et al., 1999) or in animals treated with anti-androgens (van der Schoot and Elger, 1992) the CSL persists, resulting in maldescendus testis.

Collectively, the phenotypic alterations described above suggested sex steroid insensitivity as the underlying cause of the defects observed in megalin-deficient mice. Sex steroid insensitivity was further supported by the fact that megalin-deficient embryos failed to induce regression of the CSL in response to exogenous application of androgens, a response that was readily seen in wild types (Hammes et al., 2005).

Based on the ability of megalin to internalize DBP-bound sterols, a similar function of the receptor in uptake of carrier-bound sex steroids is highly suggestive. In line with this hypothesis, megalin was shown to act as endocytic receptor for human SHBG and rodent ABP in cultured cells (Hammes et al., 2005). When exposed to sex steroids in the presence of physiological concentrations of the carriers, cells were dependent on megalin activity to internalize significant amounts of the hormones. Little steroid hormone uptake was seen in the presence of megalin antagonists or in cell types that lacked receptor expression. SHBG-bound steroids delivered by megalin entered intracellular compartments and acted as inducers of steroid target genes, the hallmark of cellular steroid action (Hammes et al., 2005).

The ability of megalin to mediate endocytic uptake of androgens and estrogens bound to carrier proteins in cultured cells suggests a working model whereby delivery of sex steroids by megalin plays a critical role in regulation of steroid-dependent maturation of the reproductive organs. Although highly suggestive, formal proof for a role of megalin in delivery of sex steroids to target tissues *in vivo* remained controversial (Rosner, 2006). The most obvious caveat is the fact that the plasma carrier for sex steroids in rodents is unknown. In humans, SHBG is produced by the liver and secreted into the circulation to act as systemic carrier for androgens and estrogens (Cousin et al., 1998; Rosner et al., 1984). In contrast in rodents, expression of SHBG is restricted to the fetal liver and to the ovaries and testes of the adult organism (Gershagen et al., 1989; Hammond and Bocchinfuso, 1996; Joseph, 1994; Joseph et al., 1997). While this expression pattern is consistent with a role of SHBG in local sex steroid metabolism in reproductive tissues, other carrier proteins are certainly involved in steroid hormone trafficking in rodents as well. Whether these carriers are ligands to megalin remains to be shown, once the identities of such carriers have been revealed. Recently, megalin-dependent uptake of fluorescent-labeled estradiol into the marginal cells of the stria vascularis in the inner ear has been shown (Konig et al., 2008). This finding strongly supports a role for the receptor as cellular entry pathway for sex steroids *in vivo*. Such uptake pathways may not only be relevant for normal functions of steroids but also contribute to the delivery of steroid hormones to androgen-dependent tumors as suggested by the association of *Megalin* gene variants with fatal outcome of prostate tumors in patients (Holt et al., 2008).

Mouse models of CBG deficiency

As well as generating mouse models with defects in presumed receptors for steroid carriers (as for megalin), one may also approach the functional significance of bound steroids by developing animal models of carrier deficiencies. According to the free hormone hypothesis, lack of the carriers should results in increased concentrations of the biologically active free hormone and, consequently, in a condition of aggravated steroid signaling.

The analysis of mouse models with induced *Dbp* gene defect has already been discussed above. Surprisingly, some phenotypes observed in this model such as protection from vitamin D toxification are incompatible with the free hormone hypothesis (Safadi et al., 1999). Similarly, phenotypic characterization of mice lacking CBG revealed some surprising findings. CBG is the major transport protein for glucocorticoids in plasma of mammalian species with more than 90% of circulating corticosteroid molecules being bound by this carrier (Breuner and Orchinik, 2002; Hammond et al., 1987; Rosner, 1990). CBG is a 55 kDa monomeric glycoprotein that is mainly secreted by the liver, but is also produced in lung, kidney, and testis (Hammond et al., 1987). CBG plays an important role in the metabolism and action of glucocorticoids. Most notably, its role in transport of otherwise insoluble glucocorticoids is appreciated. However, the identification of surface binding sites for CBG in a number of steroid-target tissues including liver, endometrium, and spleen (Hryb et al., 1986; Maitra et al., 1993; Singer et al., 1988; Strel'chyonok and Avvakumov, 1991) suggested the existence of membrane receptors for cellular uptake and/or transmembrane signaling of CBG/steroid complexes. To test the relevance of CBG for glucocorticoid action and adrenocortical stress response mice genetically deficient for CBG were generated (Petersen et al., 2006). Absence of CBG resulted in lack of corticosterone binding activity in serum and in a ~10-fold

increase in free corticosterone levels in CBG null mice, consistent with its role in regulation of circulating free hormone levels. Surprisingly, *Cbg*$^{-/-}$ animals did not present with features seen in organisms with enhanced glucocorticoid signaling. Rather, the mice exhibited increased activity of the pituitary axis of hormonal control, normal to decreased levels of gluconeogenetic enzymes, as well as an aggravated response to septic shock, indicating an inability to appropriately respond to the excess free corticosterone in the absence of CBG (Petersen et al., 2006). Thus, these data suggest an active role for CBG in bioavailability, local delivery, and/or cellular signal transduction of glucocorticoids that extends beyond a mere function as cargo transporter.

Perspective

Conceptually, the quest for receptors involved in cell type specific delivery of sterols has mainly focused on the search for membrane-associated forms of nuclear hormone receptors. Such receptor pathways have been documented for progestins (Ferrell, 1999; Zhu et al., 2003a; Zhu et al., 2003b) and estrogens (Song and Santen, 2006). However, specificity in steroid targeting may just as well be achieved by surface binding sites for carrier proteins as they are unique for distinct classes of steroids. Such a concept has evolved for cell-type specific uptake of cholesterol in lipoproteins. Intriguingly, megalin-mediated internalization of carrier bound steroid hormones follows the same basic principal, suggesting the evolutionary conservation of sterol uptake pathways. Megalin's functions in cell type specific action of steroid hormones may represent a pathway that developed early in evolution. In *Caenorhabditis elegans*, the megalin orthologue Ce-LRP1 mediates the uptake of cholesterol into cells that convert the precursor sterol into an intracellular steroid hormone essential

for regulation of larval growth (Entchev and Kurzchalia, 2005; Matyash et al., 2004; Yochem et al., 1999).

Concluding this review, one should stress the fact that endocytic pathways for carrier-bound hormones are unlikely to account for all pleotropic effects of steroid hormones. Rather, all the experimental evidence points to the fact that such uptake pathway play an important role in certain physiological conditions when fast and efficient cell-type specific uptake of steroids may be required. Notably, such pathways may be found in tissues involved in activation of steroid hormones (e.g., proximal tubules, epididymis). Also, in instances when steroid hormone action is required during a narrow time window (as for involution of the CSL), the organism may not rely on unspecific diffusion processes for delivery of these essential regulators. Exciting in terms of medical applications is the prospect that steroid dependent tumors may use endocytic pathways for acquiring large amounts of sex steroids, and that such uptake pathways may represent novel drug targets in therapy of these common neoplastic diseases.

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FIGURE LEGENDS

Figure 1. Endocytic pathway for uptake of cholesterol.

Lipoproteins are taken up by lipoprotein receptors (LDL receptor) via binding of the apolipoprotein moiety on the lipoprotein particle. Following coated-pit endocytosis, the receptors discharge their ligands in endosomes before recycling back to the cell surface. Internalized lipoproteins are catabolized in lysosomes. There, apoproteins are degraded, while cholesterol enters the cellular membrane pool via the Endoplasmatic Reticulum (ER), is converted into steroid hormones in mitochondria, or stored as cholesterol esters in cytoplasmic lipid droplets. Exit of cholesterol from lysosomes requires the activity of Niemann-Pick disease type C protein 1 (NPC-1) and NPC-2.

Figure 2. The LDL receptor gene family.

The figure depicts the structural organization of members of the LDL receptor gene family. Among other modules, their extracellular domains are composed of clusters of complement-type repeats, the site of ligand binding, as well as β-propellers that are essential for pH-dependent release of ligands in endosomes. The cytoplasmic tails harbor recognition sites for cytosolic adaptor proteins involved in receptor trafficking. LDLr, low-density lipoprotein receptor; LRP, LDL receptor-related protein; RME-2, receptor-mediated endocytosis-2; VLDLr, very low-density lipoprotein receptor.

Figure 3: Metabolism of androgen binding protein in the epididymis.

In rodents, androgen binding protein (ABP) is secreted by Sertoli cells in the testis. In the lumen of the seminiferous tubules, the carrier binds testosterone and delivers the androgen to principal cells in the epididymis. Internalization through a yet unknown

ABP receptor enables cell type specific uptake of large quantities of bound testosterone to be converted into dihydrotestosterone (DHT).

Figure 4: Renal uptake of 25-OH vitamin D_3 bound to vitamin D binding protein.

(**A**) Two-receptor model for vitamin D binding protein (DBP) uptake in renal proximal tubular cells. Complexes of DBP and 25-OH vitamin D_3 are reabsorbed from the glomerular filtrate by association of DBP with megalin, followed by endocytic uptake. Alternatively, the steroid-carrier complexes are bound by cubilin and internalized via interaction of this receptor with megalin.

(**B**) Cellular uptake and activation of DBP-bound 25-OH vitamin D_3. Complexes of DBP and 25-OH vitamin D_3 are cleared from the glomerular filtrate by association of DBP with megalin and cubilin expressed on the apical surface of cells in the proximal tubule. Complexes are internalized by receptor-mediated endocytosis via clathrin-coated pits and delivered to endosomal compartments containing ClC-5. Endocytosis of receptor/ligand complexes is assisted by Dab2 (and possibly other trafficking adaptors) bound to the cytoplasmic tail of megalin. From endosomes, un-liganded receptors recycle back to the cell surface, while DBP is degraded in lysosomes. 25-(OH) vitamin D_3 is transported to mitochondria to be hydroxylated to 1,25-$(OH)_2$ vitamin D_3 and released into the interstitial fluid where it associates with DBP again. Intracellular transport of vitamin D metabolites is controlled by association with intracellular vitamin D binding proteins (IDBP)-1 and -2 (Wu et al., 2000).

Figure 5. Androgen-dependent regression of the cranial suspensory ligament.

(**A**) Regulation of testicular descent in the rodent embryo. At embryonic day (E) 13.5 the gonads in both sexes are positioned close to the lower pole of the kidney by two ligaments, the cranial suspensory ligament (CSL) and the gubernaculum. At E17.5, androgen-induced regression of the CSL in males enables movement of the testes by the gubernaculum towards the lower abdomen. In female embryos the CSL persists, resulting in a position of the ovaries close to the kidneys. (**B**) Urogential tracts from wild type and megalin-deficient newborn male mice. The arrowhead highlights aberrant CSL attached to the testes in *megalin*$^{-/-}$ animals. bl, bladder; go, gonads; ki, kidney; ov, ovaries; te, testis.

Figure

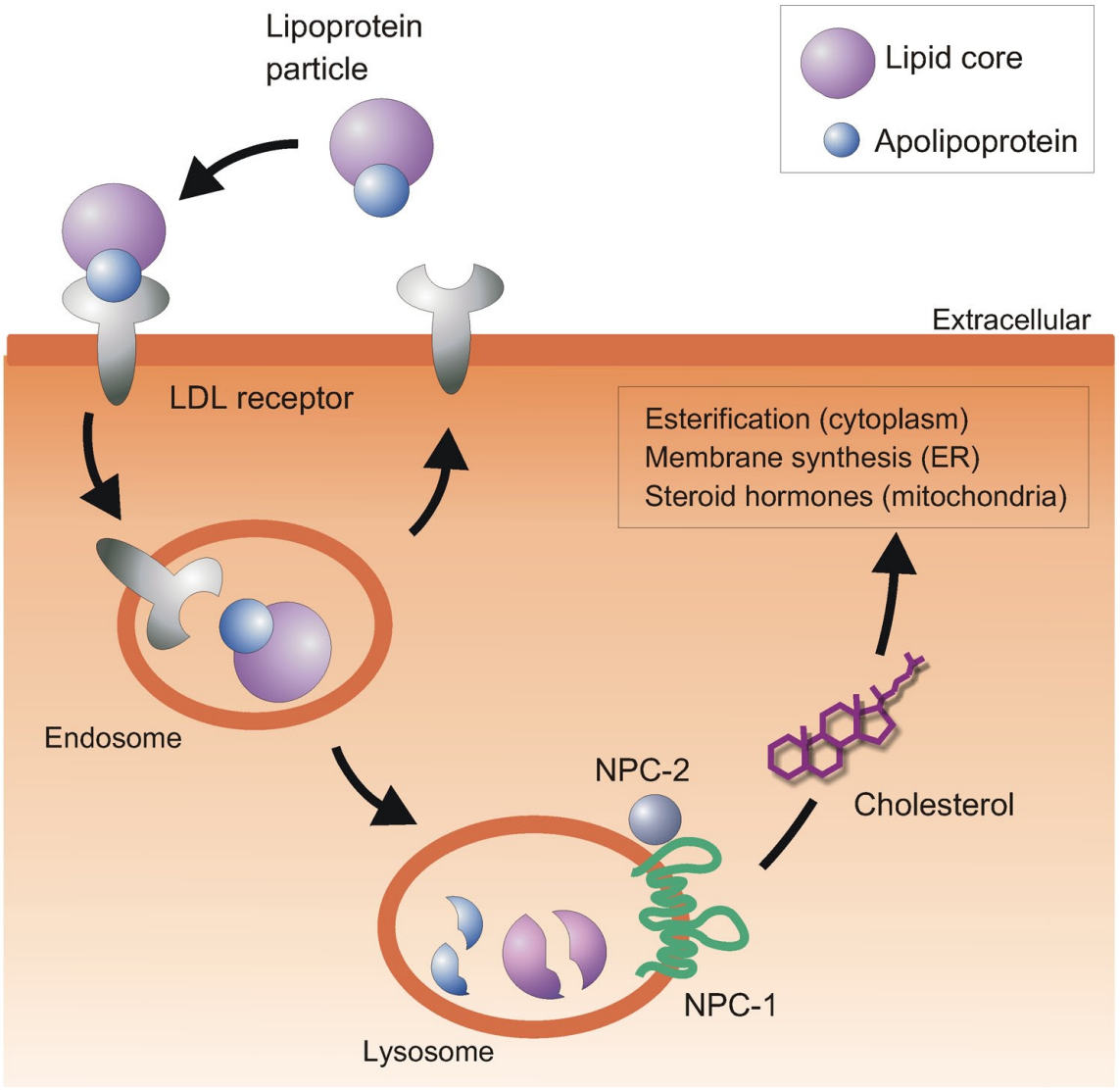

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Figure 1

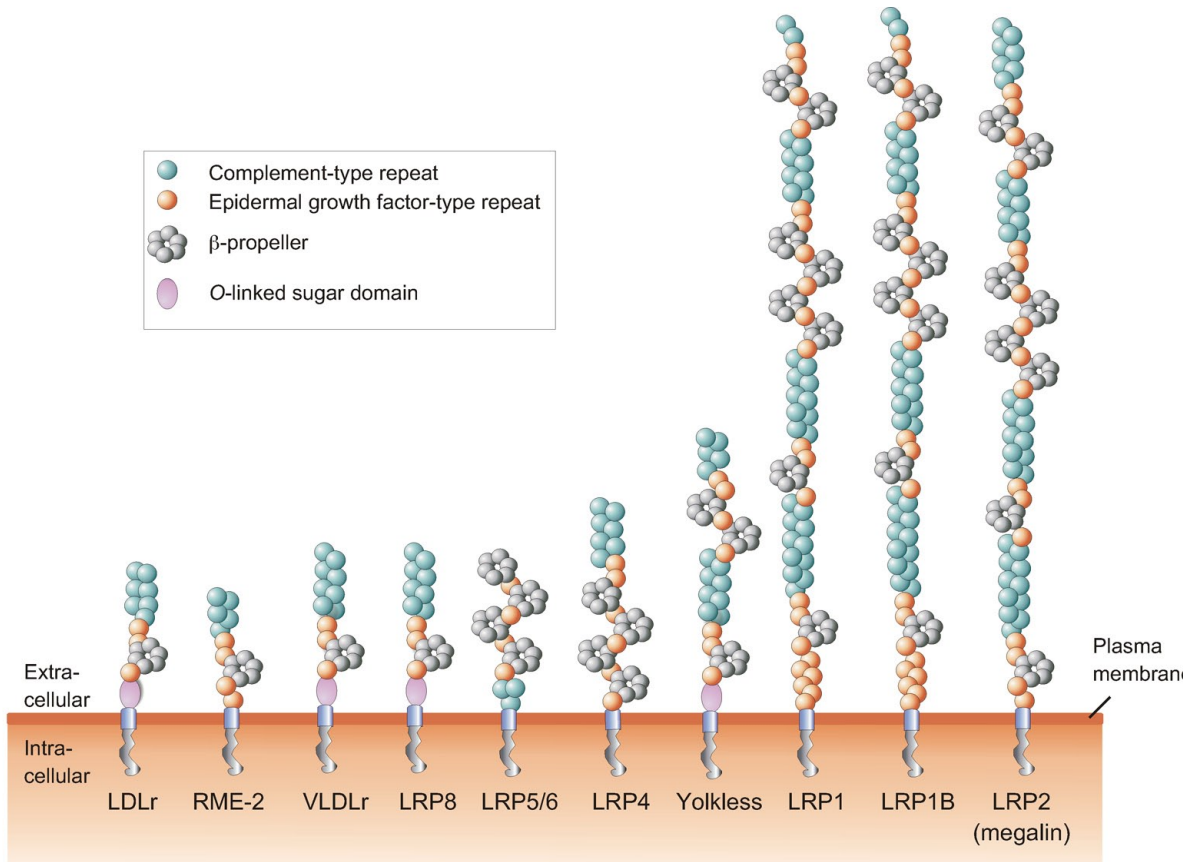

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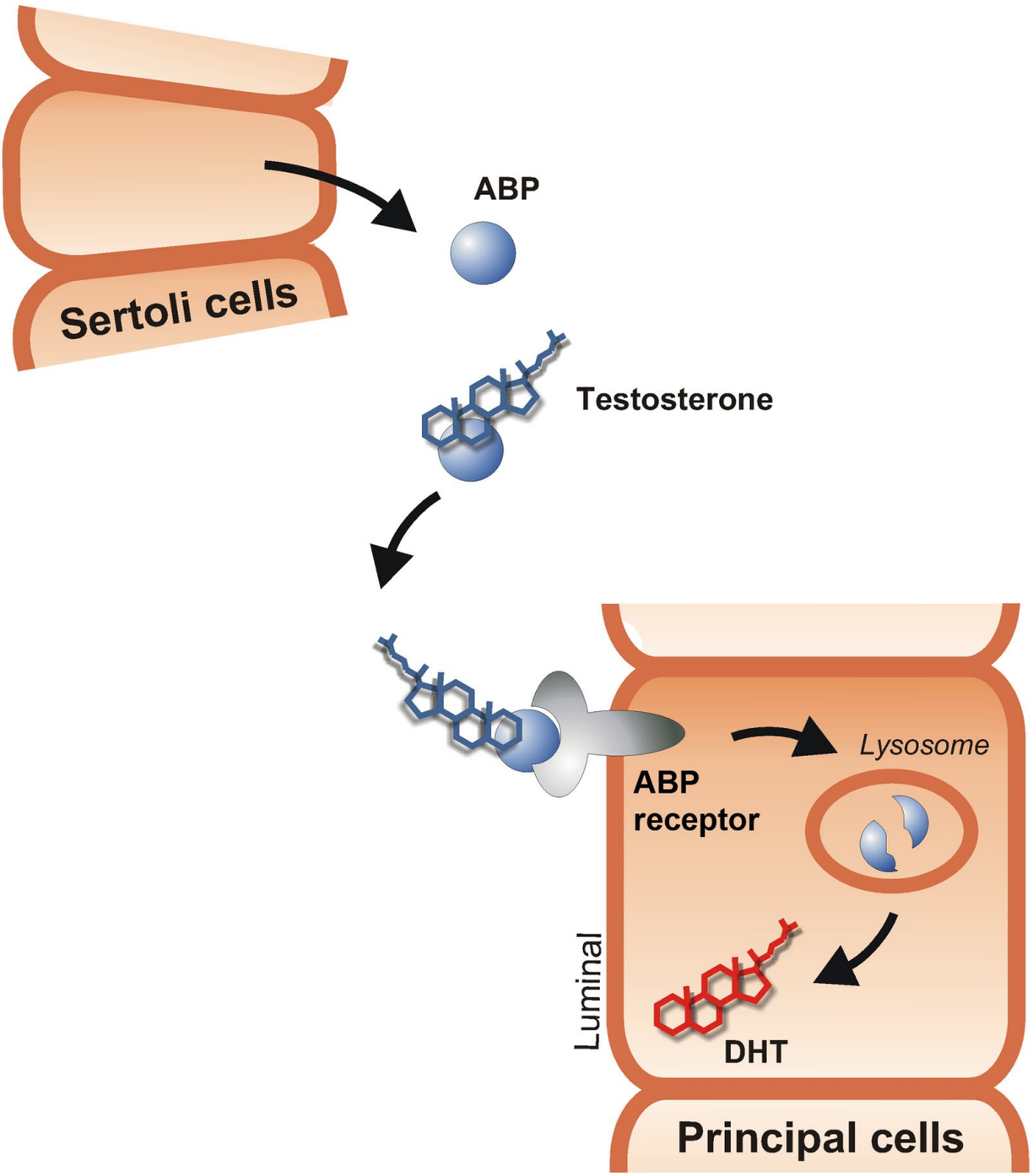
Figure 2



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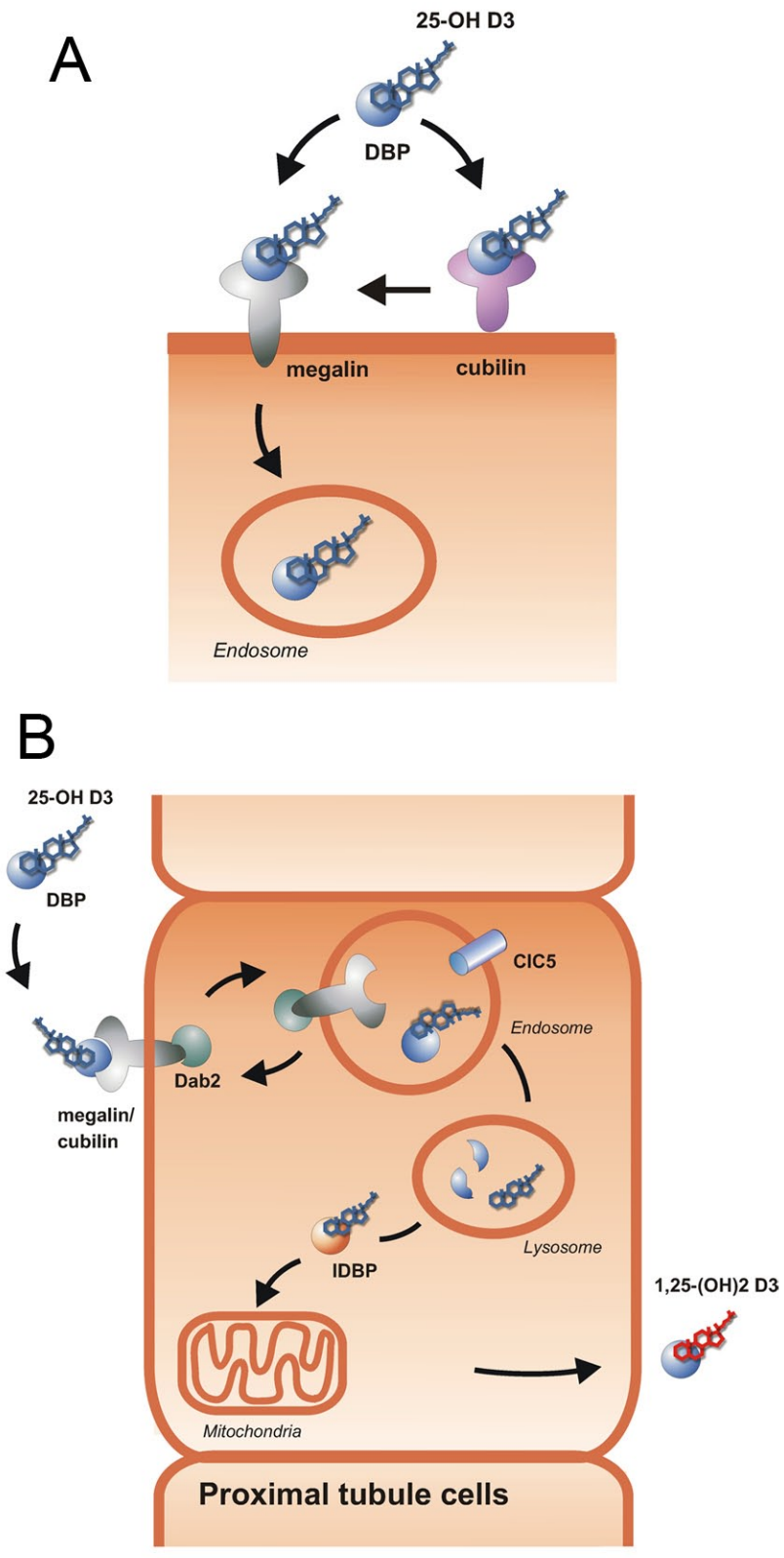

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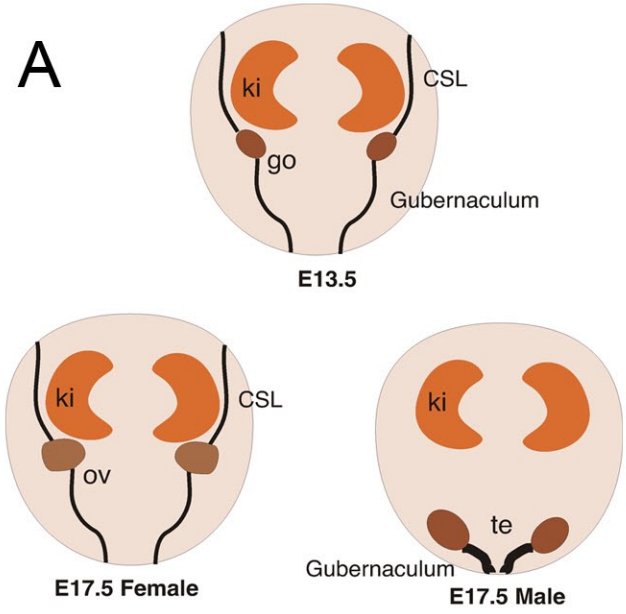
Figure 4

A



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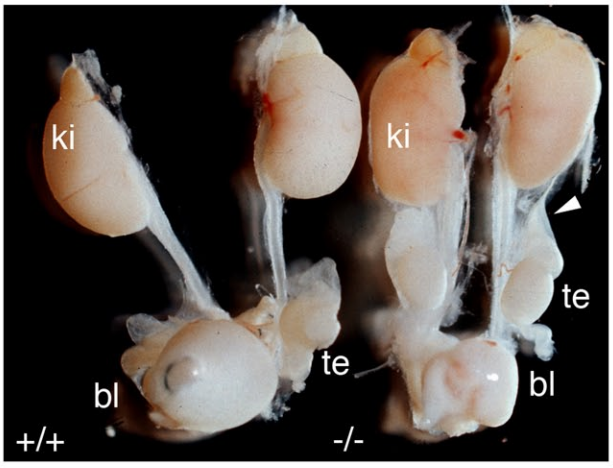

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Figure 5