

## Ghrelin and its Role in the Secretion and Motility of the Gastrointestinal Tract in Humans

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**Abstract:** *The functional plasticity of the gastrointestinal tract in the processes of digestion, transport and resorption of nutrients is carried out by complex neurohumoral factors. Tissue hormones excreted by the enteroendocrine cells ensure local regulation of the processes in the gastrointestinal wall in correspondence with the specific needs in its separate sections. One of these hormones is ghrelin. As a recently discovered hormone by Masayasu Kodzima et al. in 1999, the type of endocrine cells, which synthesize it, as well as its physiologic functions and mechanisms of operation, has not been clarified yet. Aim The aim of the present study is to establish the presence of endocrine cells of the ghrelin-producing type and the presence of ghrelin receptor GHSR-1 in the gastrointestinal tract of humans. Material and Methods Biopsy specimen from human mucosa from the stomach and duodenum is investigated by transmission electric microscope and immunohistochemical reactions for ghrelin and ghrelin receptor GHSR-1. Results Microscopically we establish the presence of several enteroendocrine cells with alleged ghrelin secretion. Ultramicroscopic features show that they are of G, D, X-like and EC1 and EC2 type. Immunohistochemically we establish ghrelin-producing cells in the gastric mucosa and ghrelin-receptor is expressed in glandular and smooth muscle cells of stomach and duodenum. Conclusion In our study we provide clinically significant data for ultramicroscopic characteristics of the potential ghrelin-producing cells in the gastrointestinal tract as well as the specific localization of ghrelin secretion in the gastric mucosa. The established positive expression of ghrelin receptor in smooth muscle and glandular cells from stomach and duodenum reveals the ability of ghrelin to influence directly the secretion and motility of these organs.*

**Keywords:** *ghrelin, gastrointestinal tract, ghrelin receptor GHS-R1*

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### I. Introduction

The functional plasticity of the gastrointestinal tract in the processes of digestion, transport and resorption of nutrients is performed by complex neurohumoral factors. Tissue hormones excreted by enteroendocrine cells ensure local regulation of the processes in the gastrointestinal wall in correspondence with the specific needs in its separate sections. Over 19 types of enteroendocrine cells, which release more than 30 types of biologically active substances with hormonal action, have been found so far. The localization of these cells in the gastrointestinal tract depends on the functions of the respective section. In the stomach and proximal portion of the small intestine, where the precise coordination of the secretory, resorptive and motor functions is necessary, the concentration of the various types of enteroendocrine cells is highest. Enteroendocrine cells are differentiated in the gastro-enteropancreatic endocrine system, part of the diffuse neuroendocrine system. The classification of enteroendocrine cells in this system is based on the ultrastructural type of secretory hormones and types of hormones which they produce. The first classification was approved in Wiesbaden in 1969 and includes 7 types of cells, the second in Bologna in 1973- 10 types, the third in Lozana in 1977-15 types. The last classification was approved in Santa Monica in 1980 and includes 19 types of endocrine cells. The difficulties in the classification stem from the fact that some enteroendocrine cells secrete more than one peptide or biogenic amine and in other cases, 2 different endocrine cells form one and the same substance (Solcia et al., 1981; Lechago, 1987;

Kvetnoy et al., 1987). Gastro-entero-pancreatic endocrine system has still not been studied thoroughly in regard to the types of cells as well as in regard to its functions and role in the regulatory systems of the body. A proof for this is the discovery of the hormone ghrelin.

**Ghrelin secretion and Functions** Ghrelin is an oligopeptide of 28 amino acid residues isolated for the first time from rat stomach. It was first identified by Masayasu Kodzima et al. in 1999, they ascertained that serum levels of ghrelin in slim rats are higher than those of fat ones (Salzet et al., 2003). Ghrelin participates in the formation of severe feeling of hunger through its connection with specific receptors in the nuclei of the diencephalon. It releases growth hormone secretion through receptors in the adenohypophysis. The root of the term 'ghre' derives from 'grow'. Metabolic effects of ghrelin are due to its function to be a ligand for the growth

hormone secretory receptor in the adenohypophysis. This receptor is of the group of G protein-coupled receptors which activate the membrane bound adenylate cyclase and the transformation of ATP into cAMP. The ghrelin receptor bears the corresponding name- growth hormone secretagogue receptor (GHS-R). A number of studies indicate that plasma levels of ghrelin increase on an empty stomach and after feeding they return to their normal levels (Tschop et al., 2000; Asakawa et al., 2001; Toshinai et al., 2001). Factors which influence ghrelin secretion are blood sugar levels, resp. insulin, and composition of the ingested nutrients. High fructose levels and low protein food content increase this secretion. Diet high in lipids decreases plasma levels of ghrelin (Dornonville et al., 2001).

Ghrelin exists in two forms- acylated and nonacylated ghrelin. 70% of serum ghrelin is in a nonacylated form. Most authors consider acylated ghrelin hormone active. Acylated ghrelin, related to GHSR-type 1a plays a major role in the regulation of somatotrophic function (Kojima et al., 1999; Lee et al., 2002). Some metabolic effects of ghrelin, however, are irrespective of its acylation. This supports the hypothesis for the existence of subtype receptors for ghrelin different from GHS-R1A (Martin-Pastor et al., 2010). There is a sexual differentiation of the plasma levels of the acetylated ghrelin. In women this level is higher than in men (Akamizu et al., 2005).

In CNS an expression of ghrelin receptors in numerous structures is established immunohistochemically. Through specific receptors in the nuclei of diencephalon, ghrelin participates in the formation of severe feeling of hunger (Broglia et al., 2004). There are scattered ghrelin immunopositive neurons in nucleus suprahypophysialis of diencephalon, tuberculum posterior of hypothalamus, nucleus posterolateralis from the lateral group of nuclei of the thalamus as well as in the antero-dorsal tegmental nuclei of mesencephalon (Hagemann et al., 2003). Ghrelin receptors are expressed in nucleus arcuatus and lateral hypothalamus (Chartel et al., 2002). In nucleus arcuatus it binds with the orexigenic neuropeptide – Y (NPY) (Fukumoto et al., 2008). In the limbic (mesolimbic) system ghrelin activates cholinergic and dopaminergic neurons which have relation to reward behavior as well as to addiction to food, alcohol, drugs (Olszewski et al., 2003; Landgren et al., 2011). Ghrelin receptors are also expressed in the hippocampus where ghrelin is related to cognitive processes, adaptation to changing conditions of the environment and the processes education and memory (Carlini et al., 2002; Senior et al., 2010). It is considered that the activation of dopamine secretion by ghrelin might be related to the treatment of neurodegenerative diseases such as Parkinson's disease (Elisabet et al., 2009).

A number of authors study the role of ghrelin in the gastrointestinal tract. There are reports in literature which reveal the relations between ghrelin and other gastrointestinal hormones- gastrin, histamine, glucagon. It is presumed it stimulates the secretion of hydrochloric acid as well as gastric emptying (Bancs et al., 2002). Ghrelin increases gastrointestinal tract motility similarly to motilin (Chartel et al., 2002). Ghrelin stimulates proliferative processes in the gastric wall and inhibits apoptosis in inflammatory conditions and oxidative stress. It increases the regenerative potential of the gastric mucosa. Its participation in anti-inflammatory mechanisms allows its therapeutic application in inflammatory conditions and diseases of the gastrointestinal tract (Peracchi et al., 2006; Sanger et al., 2008). Ghrelin regulates the exocrine function of the pancreas, has a relation to insulin secretion (Mark et al., 2011; Wierup et al., 2004). In the liver the hormone participates in the regulation of fibrogenesis in acute and chronic diseases (Walia et al., 2009).

**Ghrelin receptor** *Ghrelin receptor GHS-R1* is a G-coupled peptide cloned for the first time in 1996 from adenohypophysis and hypothalamus (Howard et al., 1996). Its binding with ligands leads to the release of growth hormone secretion. In 1999, after its discovery, ghrelin was identified as an endogenous ligand congenial to this receptor (Moreno et al., 2010). Two isoforms of the ghrelin receptor have been synthesized: GHS-R1A and GHS-R1B. Through the use of Bioluminescence Resonance Energy Transfer (BRET), heterodimers of GHS-R1A and GHS-R1B are shown during a process of oligomerisation in the endoplasmic reticulum of isolated subcellular fractions (Chow et al., 2012). GHS-R1A is a mature polypeptide of 366 amino acid sequences and 5 transmembrane domains (Albarrán-Zeckler, 2013).

In the last years ghrelin receptors are sought outside the central nervous system. In GIT ghrelin receptors have been found in rat and guinea pig. Kitazawa et al., (2011) identify ghrelin receptor GHS-R1A in vegetative afferent nerve fibres and smooth muscle cells from stomach, small and large intestine of a guinea pig. In isolated sections of these organs ghrelin causes marked contractions of the muscles of the stomach and large intestine and slighter ones in the sections of the small intestine.

Detailed studies of ghrelin and its receptors lead to the creation of ghrelin analogues, agonists and antagonists for its receptors. Peptide analogues of ghrelin EP01572 and its agonists such as Capromorelin (RQ-00000005) and TZP-101 are studied in relation to the treatment of anorexia and cancer cachexia and in adults. Clinical studies of ghrelin agonist Anamorelin in patients with decreased appetite, weight loss and symptoms related to delayed gastric emptying and constipation have been performed. Ghrelin receptor antagonists have therapeutic

applications in the treatment of metabolic disturbances, obesity, and diabetes type 2. The data established in literature from the studies related to ghrelin receptor are still not conclusive. A full map of the distribution of ghrelin receptors in CNS has not yet been outlined.

This would shed more light on the participation of ghrelin in the functioning of the different functional systems in CNS. The presence of these receptors in the peripheral tissues means that ghrelin participates in the regulation of the metabolic processes not only indirectly, through the growth hormone, but ghrelin itself has direct hormonal effects on the target cells. The presence of ghrelin receptors in the gastrointestinal tract in glandular and smooth muscle cells would mean that ghrelin, as other intestinal hormones, apart from endocrine, also has paracrine effect and participates in the local regulation of secretory processes and motility of GIT.

**Ghrelin-producing cells** Through immunohistochemistry and insitu hybridization and RT-PCR ghrelin is found in endocrine cells from the gastric mucosa, tunica mucosa and islets of Langerhans in the pancreas of an adult (Dornonville et al., 2001; Svenson et al., 2002; Grönberg et al., 2008). In rodents ghrelin-producing cells are observed also in the other sectors of the GIT – duodenum, ileum, colon, cecum. However, the main production of ghrelin in the mature animal and human organism is in the gastric mucosa. Following experimental gastrectomy of rats their plasma levels of ghrelin decrease with 80% (Dornonville et al., 2001). The same results are obtained also in patients with operative gastrectomy. In them, plasma levels of ghrelin decrease with 65% (Ariyasu et al., 2001).

So far there is no definitive answer to the question which type of endocrine cells produce this hormone. Whether it is synthesized in a specific enteroendocrine cell of a new, unknown so far, type? Whether it is produced by one, but already familiar type of enteroendocrine cell? The possibility that different types of enteroendocrine cells synthesize ghrelin, along with other hormones familiar to them, also exists. Depending on the morphology and hormonal activity of cells, according to some authors, it is produced by cell type A (glucagon), according to other type D (somatostatin), G (gastrin), ECL (histamine), (Date et al., 2000, Svenson et al., 2002; Sakata et al., 2010). Others consider X cells, with unknown function so far, to be ghrelin – producing (Rindi et al., 2002), and still others consider this hormone to be produced by another, unknown type of cells (Svenson et al., 2002; Yabuki et al., 2004). There are authors who have changed their opinion with time. Studying ghrelin secretion from isles of Langerhans in pancreas, in older reports Wierup et al., (2002, 2004) claim that ghrelin is produced by a separate type EE cell. In later reports these authors account that one and the same pancreatic endocrine cell secretes both ghrelin and other hormones (Wierup et al., 2013).

### **Aim**

The aim of the present study is to establish the presence of enteroendocrine cells from the ghrelin-producing type and the presence of ghrelin receptor GHSR-1 in the gastrointestinal tract of humans.

## **II. Material and Methods**

Our study was performed with biopsy specimen from the mucosa of human stomach and duodenum. Biopsy specimen from stomach: corpus et antrum gastricum; and from duodenum, pars superior duodeni is obtained through fibrogastrosopic study of 15 female patients aged 45- 72 years from the Clinic of Gastroenterology at UMHAT ‘St.George’ and MHAT ‘St. Pantheyleymon’ in the town of Plovdiv. These patients lack endoscopic data for pathological changes in the gastric mucosa. We performed transmission electron microscopic study (TEM) and immunohistochemical study (IHC) of ghrelin and ghrelin receptor GHSR-1. Biopsy material for TEM is fixated in 4% glutaraldehyde NaPO<sub>4</sub> buffer 0,1M, with subsequent postfixation in 4% OsO<sub>4</sub> and 0,2M S’collin buffer. It is dehydrated in ascending alcohol series and is included in durcupan. It is kept at 56°C for 48h in gelatin capsules. Gelatin capsules are removed with warm water and after that the material is cut in thick sections - 0,5 mm. The sections are mounted on glass slides, stained with methylene blue, covered and observed in a light microscope to determine the area for cutting. What follows is the cutting of ultrathin sections with 300 nm thickness and their mounting on platinum nets. The mounted cuts are contrasted in 5% uranyl acetate, diluted 1:1 with 100% alcohol for 80 min. in a dark place. They are washed in 50% alcohol and Reinold’s solution (aqueous solution of Pb (NO<sub>3</sub>)<sub>2</sub>, Na (CoH<sub>5</sub>O<sub>7</sub>) 2H<sub>2</sub>O for 20 min. and bid H<sub>2</sub>O. Observation and microphotographs are performed on TEM ‘Philips CM 12’.

Immunohistochemical reactions were performed according to the ABC method through rabbit ABC Staining System (Santa Cruz Biotechnology, USA) with the respective primary antibody (Table № 1.).

<b>Antibody</b>	<b>Catalogue number</b>	<b>Dilution in PBS</b>	<b>Localisation of expression</b>	<b>Granule colour</b>
<b>Ghrelin</b>	goat polyclonal ghrelinantibody (C-18): sc-10368 - Santa Cruz Biotechnology USA	1:100	cytoplasmic	Black granules

<b>Ghrelin receptor GHSR-1</b>	goat polyclonal antibody GHSR-1: sc-10351 - Santa Cruz Biotechnology USA	1:100	cytoplasmic	Fine brown granules
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**Table 1.** List of the primary antibodies used in the IHC test.

The biopsy material for the immunohistochemical study is fixed in a Buen solution for 24 hours and contains paraffin. Paraffin sections with a thickness of 5µm are deparaffinized and incubated for 30 min. in a 2% H<sub>2</sub>O<sub>2</sub> methanol for inactivation of endogenous peroxidase. The primary ghrelin antibody (goat polyclonal ghrelin antibody: sc-10368 - Santa Cruz Biotechnology USA) is diluted in PBS in 1:100 ratio. The primary ghrelin antibody for ghrelin receptor GHSR-1 (goat polyclonal antibody GHSR-1: sc-10351 - Santa Cruz Biotechnology USA), is diluted in PBS in 1:100 ratio. The incubation of sections with the respective antibody is performed in 4°C for 12 hours in a humid chamber. What follows is incubation with the biotinylated secondary antibody for 30 min., ABC complex for 15 minutes and visualization with DAB chromogen. The deparaffinized and mounted with Vecta Mount sections are observed under a microscope and photo documented.

We used a semi-quantitative evaluation method for the obtained results. Positive reaction for ghrelin is reported in the presence of black granules in the secretory granules in cells. Positive reaction for ghrelin receptor GHSR-1 is reported in the presence of fine brown granulation in the cell cytoplasm. The specificity of immunohistochemical reactions for each studied antigen is confirmed by negative controls in which the specific antibodies are substituted with a buffer (PBS) or normal non-immune serum. In them there is a complete lack of a product of the respective reaction. Observation and photo documentation of microscopic preparations are performed with digital photo microscopic camera of a light microscope “Olympus BX51”.

### III. Results

**TEM** study of biopsy material from gastric corpus and antrum as well as from duodenum of adults gives us the opportunity to ascertain the presence of different types of enteroendocrine cells.

**G-type enteroendocrine cells** Enteroendocrine cells of G-type hold round or oval secretory granules with a distinct limiting membrane and various electronic densities. The diameter of the granules is from 180 to 300 nm. The matrix of the secretory granules is filamentous or in small granules and between it and the limiting membrane there is light and narrow halo. In antral mucosa of the stomach prevail G cells with light transparent electronic granules, among which there are relatively few denser and smaller granules. In the duodenum the granules of G-cells are 150-250nm in size and possess higher electronic density. Enteroendocrine cells of G-type secrete mainly gastrin as well as enkephalins and endorphins. (fig. 1., fig. 2.)

**D type enteroendocrine cells** D type enteroendocrine cells have large granule sizes between 250 and 400 nm. They are homogeneous with moderate dense core. Granular membrane is interrupted. In the stage of granulopoeza complex Golgi produces small granules. Later they merge and form larger ones, with grainy contents. In the process of maturation graininess them gradually decreases. The secretory product of D cells is somatostatin (fig. 3.)

**X type endocrine cells (A-like cells)** X type enteroendocrine cells (A-like cells) have dense round granules with average diameter of 250nm. A specific feature is the presence of granules with eccentric dense core and broad halo below the granulated membrane. The secretory product is identified with certainty. Some authors compare them to A cells, secreting glucagon, and others- to F cells with unknown secretory product as well (Fig. 4.)

**EC type enteroendocrine cells** The basic morphological characteristic of serotonin-producing EC cells is based on the electron microscopic description of the serotonin granules they contain.

These are polymorphic stick-shaped or biconcave in form with narrow light halo and high electron density. According to the ultrastructural characteristic of the granules and character of the secretory products three types of EC cells are distinguished – EC1, EC2 and ECn type. EC1

cells are found mainly in the stomach. Their granules are polymorphic, most often elongated or oval in shape and sized 200-300 nm. They contain serotonin and substance P (fig. 5.). EC2 cells are localized mainly in the small and large intestine. Their cytoplasm is filled with oval or irregular in shape granules, 200-400 nm in size, containing serotonin and motilin (fig. 6.). ECn are most numerous in the duodenum. Their granules are small to moderate in size, with moderate electron density and contain serotonin.

#### Immunohistochemical study

**Immunohistochemical reaction for ghrelin** Cells with positive expression of ghrelin are ascertained only in biopsy specimen from gastric corpus. They can be observed in the single layered cylindrical secretory epithelium on areae gastricae and glands. The main gastric glands, gll. gastricae propriae, fill the loose connective tissue of lamina propria in the form of densely located, parallel tubules. Along each gland, mainly in

the area of its body, rarely in the area of the fundus, cells with presence of black granules in the cytoplasm can be observed. (Fig. 7.).

Ghrelin positive cells are situated on the basal lamina. Most of them have rounded shape and do not reach the lumen of the gland with their apical part. The region of the cell in which the nucleus is located is illuminated. The presence of ghrelin is ascertained through fine black granulation localized in the basal part of the cytoplasm. These are cells from 'closed' type which have mainly endocrine activity (fig. 8.). Other ghrelin-positive cells have conical shape. Their narrowed apical part reaches the lumen of the gland. A nucleus located above a narrow basal section is outlined which is coloured intensely in black from the accumulation of secretory granules in it. These are probably cells from an 'open' type (fig. 9). Ghrelin expression is ascertained in single cells located outside the glands. They are situated near the fundus of some glands but below the basal lamina in the underlying loose connective tissue (fig. 10).

**Immunohistochemical reaction for ghrelin receptor GHS-R1** Immunohistochemical study of ghrelin receptor GHS-R1 ascertains its positive expression in biopsy specimen from the three localities- corpus, antrum and duodenum. In the corpus mucosa, the receptor is visualized through the presence of brown granulation in some cells of the main glands. The number of these cells is not large. They are located mainly in the central parts of the glands, irregularly among the other epithelial cells (fig. 11.). In biopsy specimen from antrum immunohistochemical reaction for ghrelin receptor GHS-R1 is positive in smooth muscle cells. Whole sheaves of leiomyocytes located in different directions exhibit brown granulation which spreads throughout the cell (fig. 12.). Ghrelin receptor GHS-R1 is expressed in biopsy specimen from duodenum as well. Fine brown granulation is observed in the epithelial cells of the absorptive epithelium of the villi (fig. 13.) as well as in the glandular cells of crypts of Lieberkuhn (fig. 14.).

#### **IV. Discussion**

Our study ascertains ghrelin expression in endocrine cells from the main glands in the gastric corpus of humans. Ghrelin-producing cells are located mainly in the region of the body of the glands and more rarely in their fundus. In regard to morphology we ascertain the presence of ghrelin-producing cells from the 'open' and 'closed' type. This data of ours coincides with ghrelin-producing cells in the various sections of GIT of rat observed by Sakata et al., (2002). In rat stomach cells from 'closed' type predominate in contrast to the other sections of GIT such as duodenum and ileum where cells from the 'open' type predominate (Sakata et al., 2002; Zhao et al., 2008).

Despite the numerous studies on ghrelin, there is no consensus on the type of cells which secrete it. Some authors claim that ghrelin is produced by enteroendocrine cells A type (glucagon), according to others it is produced by D cells (somatostatin), G type (gastrin), ECL type (histamine) (Sakata et al., 2010). Still other researchers consider that it is enteroendocrine cells from X-type (A-like cells), which are so far with unknown function, that are ghrelin-producing cells (Rindi et al., 2002; Peeters, 2005). There are also advocates of the hypothesis that this hormone is produced by new, unknown so far, types of enteroendocrine cells (Svenson et al., 2002; Yabuki et al., 2004). The ghrelin-producing cells found in the pancreas are referred to as epsilon cells (Andralojc et al., 2009). We believe that it is more likely that ghrelin is synthesized by enteroendocrine cells that are already familiar since it has been ascertained that numerous cells from the Gastroenteropancreatic Endocrine System system produce more than one hormone. The most likely candidates for ghrelin-producing cells are precisely enteroendocrine cells of G-, D-, X-, and EC- type.

Recently data on the presence of ghrelin receptors outside the central nervous system has begun appearing. There is a small number of reports on the immunohistochemical expression of GHS-R1 in muscle and epithelial cells in the digestive, reproductive and cardio-vascular systems.

We establish GHS-R1 expression in smooth muscle cells from the antral section of the gastric wall in humans. Similarly to our study, Kitazawa et al. (2011) report of presence of GHSR1 in GIT of rat and guinea pig, through which the gastro-intestinal motility is affected.

In the gastric corpus of adults we find immunohistochemical expression of GHS-R1 ghrelin receptor in the epithelial cells of main glands. In the duodenum ghrelin receptor expression we observe both in epithelial cells of the covering epithelium and in the crypts of Lieberkuhn.

In similarity to our study GHS-R1a is found in endothelial cells. For the first time Li et al., (2007) demonstrate immunohistochemical expression of GHS-R1 in human endothelial cells from the microcirculatory system. In vivo and In vitro ghrelin stimulates cellular proliferation, migration and angiogenesis.

In the use of medications, analogues of ghrelin, agonists and antagonists for its receptors, the broad spectrum of functions in which ghrelin participates should be born in mind. The presence of ghrelin receptors in the central nervous system as well as in the peripheral tissues implies the adverse effects of these medications.

Functional diseases of GIT with their leading symptoms such as diarrhea, constipation, nausea, vomiting and pain are a serious problem in contemporary gastroenterology. The possibility for ghrelin to interfere with the complex pathogenic processes in them is too strong bearing in mind the possibility for its direct effect on the glandular and smooth muscle cells through its receptors. In the treatment of inflammatory diseases of GIT we should not ignore the role of ghrelin in regenerative processes through its receptor-mediated participation in cellular proliferation and regeneration.

Ghrelin participation in metabolic disorders, leading to cachexia or obesity, is indisputable which reveals the necessity for its study in relation to new therapeutic approaches.

## V. Conclusion

In our study we present data significant for clinical practice related to ultramicroscopic characteristic of the potential ghrelin-producing cells in the gastrointestinal tract as well as the precise location of ghrelin secretion in the gastric mucosa. The ascertained positive expression of ghrelin receptor in smooth muscle and glandular cells from stomach and duodenum reveal the ability of ghrelin to affect directly the secretion and motility of GIT. This data is necessary in seeking new treatment approaches in numerous diseases such as those leading to cachexia, such as anorexia, consumptive thrombohemorrhagic syndrome in cancer patients and the elderly; as well as in metabolic disorders accompanied by obesity.

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