# 13

# Septins and apoptosis

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## INTRODUCTION

Playing major roles in the cell, septin proteins are phylogenetically conserved among diverse eukaryotes ranging from fungi to *C. elegans* all the way to mammals (Kinoshita, 2003 and see Chapter 2). All septin family members share a preserved P-loop GTP•-binding domain, yet contain large sequence variability and diverse motifs (Kinoshita, 2003; Saraste, Sibbald and Wittinghofer, 1990; Bourne, Sanders and Mccormick, 1991; Hall and Russell, 2004). Accordingly, septin proteins are shown to participate in many key cellular functions including vesicle trafficking, cytoskeletal and filamental formation, membrane remodelling and exocytosis (Lindsey and Momany, 2006; Hall *et al*., 2005; Spiliotis and Nelson, 2006; Hall and Russell, 2004; Kartmann and Roth, 2001). Though Septins are involved in a large variety of fundamental cell processes, a direct involvement of a family member in programmed cell death, apoptosis, was reported only in 2000, with the discovery of SEPT4_i2[1] (formerly known as *ARTS protein*; Larisch *et al*., 2000; Larisch-Bloch *et al*., 2000). In this chapter we will describe the cellular and biochemical characteristics of apoptosis and concentrate on the so far only reported pro-apoptotic septin family member SEPT4_i2. We will illustrate the genetic and biochemical basis for involvement of the SEPT4_i2 in apoptosis.

[1]In line with HGNC guidelines and the emerging consensus on septin nomenclature (see Appendix 2) the SEPT4 isoform formerly known as *ARTS* will be designated hence forth SEPT4_i2, the product of the *SEPT4_v2* transcript of the *SEPT4* gene.

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## APOPTOSIS: A PROGRAMMED CELL DEATH MECHANISM

Essentially all animal cells have the ability to kill themselves by activating an intrinsic cell suicide program when they are no longer needed or have become seriously damaged (Vaux and Korsmeyer, 1999; Jacobson, Weil and Raff, 1997). The execution of this program leads to a morphologically distinct form of cell death termed *apoptosis* (Kerr, Wyllie and Currie, 1972; Wyllie, 1980). It is now generally accepted that apoptosis is of central importance for development and homeostasis of metazoan animals. The roles of apoptosis include the sculpting of structures during development, deletion of unneeded cells and tissues, regulation of growth and cell number, and the elimination of abnormal and potentially dangerous cells (Jacobson, Weil and Raff, 1997). In this way, apoptosis provides a stringent and highly effective 'quality control mechanism' that limits the accumulation of harmful cells, such as self-reactive lymphocytes, virus-infected cells and tumour cells (Naik, Karrim and Hanahan, 1996; Reed, 1995; Thompson, 1995; White, 1996). On the other hand, inappropriate apoptosis is associated with a wide variety of diseases, including AIDS, neurodegenerative disorders and ischemic stroke (Martinou *et al*., 1994; Pettmann and Henderson, 1998; Thompson, 1995; Raff, 1998).

The main executioners of apoptosis are a set of cysteine proteases called *caspases* (for cysteine aspartase), that are widely expressed as inactive zymogens (Nicholson and Thornberry, 1997). These caspase zymogens are converted to the active protease as cells are selected to die. Once activated, caspases are thought to cleave a variety of important structural proteins, enzymes and regulatory molecules which are essential for the proper function of the cell (Thornberry and Lazebnik, 1998). Generally, caspases are divided into two classes based on their function; *effector caspases* which cleave protein substrates and execute the apoptosis program (such as caspases 3, 6 and 7) and *initiator caspases* which cleave inactive pro-forms of effector caspases, thereby activating the effector caspases, leading to the death of the cell (such as caspases 8, 9, 10 and 2) (Kumar, 1995; Salvesen and Dixit, 1997; Thornberry, Rosen and Nicholson, 1997). Many different signals that can originate either from within the doomed cell or from its extracellular environment can trigger apoptosis (Steller, 1995). These signals include steroid hormones, peptide survival factors, cell adhesion, specific cell surface receptors, viral infection, oxidative stress, excitotoxicity, ischemia, unfolded proteins and unrepaired DNA breaks (such as caused by ionizing radiation) (Truman,Thorn and Robinow, 1992; Oppenheim, 1991; Raff, 1992; Pettmann and Henderson, 1998; Nagata, 1997; Bergmann *et al*., 1998).

Two main signalling pathways transmit the death signals leading to the programmed cell destruction; the '*extrinsic* and the *intrinsic pathway*'. The *extrinsic pathway* is triggered by binding of ligands to specific cell surface death receptors

such as Fas, TNF1 and TRAIL (Wallach *et al*., 1997; Srinivasula *et al*., 1996; Golstein, 1997). The second pathway and most abundant in cells, is the *intrinsic* or *mitochondrial pathway* which when stimulated by various stress or cytotoxic cellular signals results in the release of apoptosis promoting factors including cytochrome c from the mitochondria (Green and Kroemer, 2004). The *Bcl-2* (B-cell lymphoma 2) family of proteins govern mitochondrial outer membrane permeabilisation and can be either pro-apoptotic (Bax, Bak and Bok among others) or anti-apoptotic (including Bcl-2, Bcl-xL and Bcl-w, among an assortment of others).

There are a number of theories concerning how the Bcl-2 gene family exerts their pro- or anti-apoptotic effect. A most common hypothesis states that this is achieved by activation or inactivation of an inner mitochondrial permeability transition pore, which is involved in the regulation of matrix $Ca^{2+}$, pH and voltage. It is also thought that some Bcl-2 family proteins can induce (pro-apoptotic members) or inhibit (anti-apoptotic members) the release of cytochrome c into the cytosol which, once there, activates caspase-9 and caspase-3, leading to apoptosis. Due to their ability to receive, coordinate and dispatch death signals, mitochondria serve as a central junction of cellular decisions ranging between cellular survival and demise. In healthy normal cells, unwanted apoptosis is prevented through the action of a set of proteins termed *inhibitors of apoptosis proteins* (IAPs), which bind active caspases during non-apoptotic conditions thereby inhibiting their function (Salvesen and Duckett, 2002). IAPs were first identified as baculovirus proteins that inhibit apoptosis in infected insect cells (Clem, Fechheimer and Miller, 1991; Clem and Miller, 1994) and were later found to be largely distributed in metazoan cells. All IAP proteins contain between one to three baculovirus repeat domains (BIR) which directly interact with caspases resulting in inhibition of their protease activity. Some of the IAP proteins also contain a RING domain bearing an E3-ubiquitin ligase function (Hay, Wassarman and Rubin, 1995; Roy *et al*., 1997; Rothe *et al*., 1995; Roy *et al*., 1995; Duckett *et al*., 1996). Under apoptotic conditions active caspases become available for promoting cell destruction through their release from binding to IAPs and activation within the *apoptosome* complex. The apoptosome is a cytosolic complex created once cytochrome c exits the mitochondria and binds an adaptor protein termed APAF-1, which in the presence of dATP recruits multiple pro-caspase 9 and induces its processing into active caspase 9 molecules. This high molecular weight complex can then activate the effector caspases-3 and -7 leading to the final disintegration of the cell (Thornberry and Lazebnik, 1998). Importantly, during apoptosis the caspase inhibition exerted by IAPs is lifted by a set of proteins termed *IAP-antagonists* which are released from mitochondria enabling them to bind IAPs in the cytososl and unleash caspase activity. The best known IAP-antagonists are Smac/Diablo and Omi/HtrA2 (Du *et al*., 2000; Suzuki *et al*., 2001).

## DIRECT INVOLVEMENT OF SEPTINS IN APOPTOSIS

### *SEPT4 transcripts and isoforms*

The SEPT4_v2 transcript encodes SEPT4_i2 (formerly known as *ARTS*). This is so far the only septin directly involved in promoting apoptotic cell death. SEPT4_i2 was discovered following a non-biased genetic screen using retroviral insertional mutagenesis approach on NRP-154 rat prostate epithelial cells specifically responding in apoptosis to TGF-β treatment. The screen yielded a 1.8 kb cDNA, that mapped to the human chromosome 17q22-23 and was revealed to be a splice variant of the *SEPT4* gene (Larisch-Bloch *et al*., 2000; Larisch *et al*., 2000). Several structural differences exist between SEPT4_i2 and the other splice variants of the *SEPT4* gene; First, SEPT4_i2 lacks 20 amino acids at its N′-terminus, which are part of most other *SEPT4* splice variants. Second, SEPT4_i2 lacks the coiled-coil domain at its C′-terminus which is thought to play a role in intermolecular interactions (Sheffield *et al*., 2003). Though sharing the conserved GTP-binding domain, SEPT4_i2 lacks the G4 domain which may result in its inability to exchange GDP/GTP (Larisch *et al*., 2000). Most importantly, SEPT4_i2 contains a unique stretch of 27 amino acid caboxy terminus not found in any other Septin, or any other yet reported protein. This unique sequence of SEPT4_i2 is responsible for its unusual pro-apoptotic function which is atypical to the Septin family members. This unique sequence became a part of the SEPT4_i2 splice variant following an intron retention event occurring in the *SEPT4* gene.

*Intron retention* is defined by the presence of a transcript-confirmed intron within a transcript-confirmed exon. Therefore, mRNAs for a functional protein include a retained intron code which differ from the protein isoforms translated from the non retained mRNA (Kondrashov and Koonin, 2003; Galante *et al*., 2004). In the case of SEPT4_i2, intronic sequences originally spaced between exon VI and VII of the *SEPT 4* gene, turned into exonic sequence delineating the new SEPT4_i2 isoform containing unique C-terminus sequence (Larisch *et al*., 2000).

SEPT4_i2 also differs from most other Septin in its cellular localization. SEPT4_i2 is mainly localized in mitochondria of normal cells (Larisch *et al*., 2000), as opposed to the typical localization of other septins to actin stress fibres and cytoskeletal structures (Kinoshita *et al*., 1997; Joberty *et al*., 2001; Peng *et al*., 2002; Hsu *et al*., 1998; Kinoshita, 2003; Kinoshita, Noda and Kinoshita, 2000). One other mouse septin, M-Septin, was reported to be localized in mitochondria, yet no indication for involvement of M-Septin in apoptosis has been reported (Takahashi *et al*., 2003). Though four different splice variants of the human *SEPT4* gene are described, careful analysis of the data reveals that only two experimentally validated transcripts are reported; variant one, *SEPT4_v1* also known as; PnutL2, H5, hCDCREL-2, MART, CE5B3, Bradeion beta and variant two, *SEPT4_v2 that encodes* SEPT4_i2. (or ARTS). The third variant, *SEPT4_v3* shown in NCBI Genbank is found thus far merely as a computational prediction

with no experimental confirmation of its existence. A fourth hypothetical isoform is Bradeion α (Tanaka *et al*., 2002; Tanaka *et al*., 2001; Tanaka *et al*., 2003).

Two versions of Bradeion were reported; Bradeion Alpha and Bradeion Beta. While Bradeion Beta cDNA sequence is identical to the sequence of all other *SEPT4_v1* transcripts, Bradeion Alpha (NM_080417.1) which appeared in the past as GenBank accession number AB002110.1 was recently deleted from NCBI sequences since it was found to be a nonsense-mediated mRNA decay (NMD) candidate. NMD transcripts are degraded quickly and most likely do not encode a protein product. Figure 13.1a shows a schematic representation of the currently identified human *SEPT4* gene splice variants as presented in the NCBI database and substantiated by scientific reports. Taking into consideration the current experimental knowledge available, it seems that the human *SEPT 4* gene encodes only two distinct protein variants. Independent and thorough corroboration of this is required.

### *Mechanism of SEPT4_i2 induced apoptosis*

Evidence that SEPT4_i2 plays a role in apoptosis has come both from gain and loss of function studies. In certain cells, elevated levels of SEPT4_i2 is sufficient to induce apoptosis (Gottfried *et al*., 2004; Lotan *et al*., 2005). More generally, expression of SEPT4_i2 can promote apoptosis in response to a variety of pro-apoptotic stimuli such as, Fas, TGF-beta, cytosine arabinoside, etoposide and staurosporine (STS). Conversely, down-regulation of endogenous SEPT4_i2 by anti-sense expression was shown to protect cells against TGF-beta-induced apoptosis (Larisch *et al*., 2000). In all these cases, SEPT4_i2-mediated cell killing leads to caspase activation. SEPT4_i2 is found to induce activation of initiator caspase-9, as well as activation of the main effector caspase-3 (Gottfried *et al*., 2004; Lotan *et al*., 2005; Elhasid *et al*., 2004).

Because SEPT4_i2 is implicated in a wide variety of apoptotic paradigms, it seems to function at a central apoptotic junction where different upstream apoptotic inputs converge to mediate caspase activation. The main mechanism by which SEPT4_i2 exerts its apoptotic activity is through direct binding and inhibition of IAPs (inhibitors of apoptosis proteins). Upon induction of apoptosis, SEPT4_i2 is released from mitochondria and co-localizes with XIAP (X-linked-IAP) in the cytosol. Binding of SEPT4_i2 to XIAP is direct, as recombinant SEPT4_i2 and XIAP proteins can bind to each other *in vitro* (Gottfried *et al*., 2004). SEPT4_i2 binding to XIAP is specific and related to its pro-apoptotic function, as mutant forms of SEPT4_i2 and other related but non-apoptotic Septins fail to bind XIAP and fail to induce apoptosis. In addition, binding of SEPT4_i2 to XIAP causes a significant reduction in XIAP levels and leads to caspase activation and cell death (Gottfried *et al*., 2004).

The mitochondrial localization of SEPT4_i2 and its ability to bind XIAP are shared with other mammalian IAP-antagonists, most known ones are Smac/Diablo

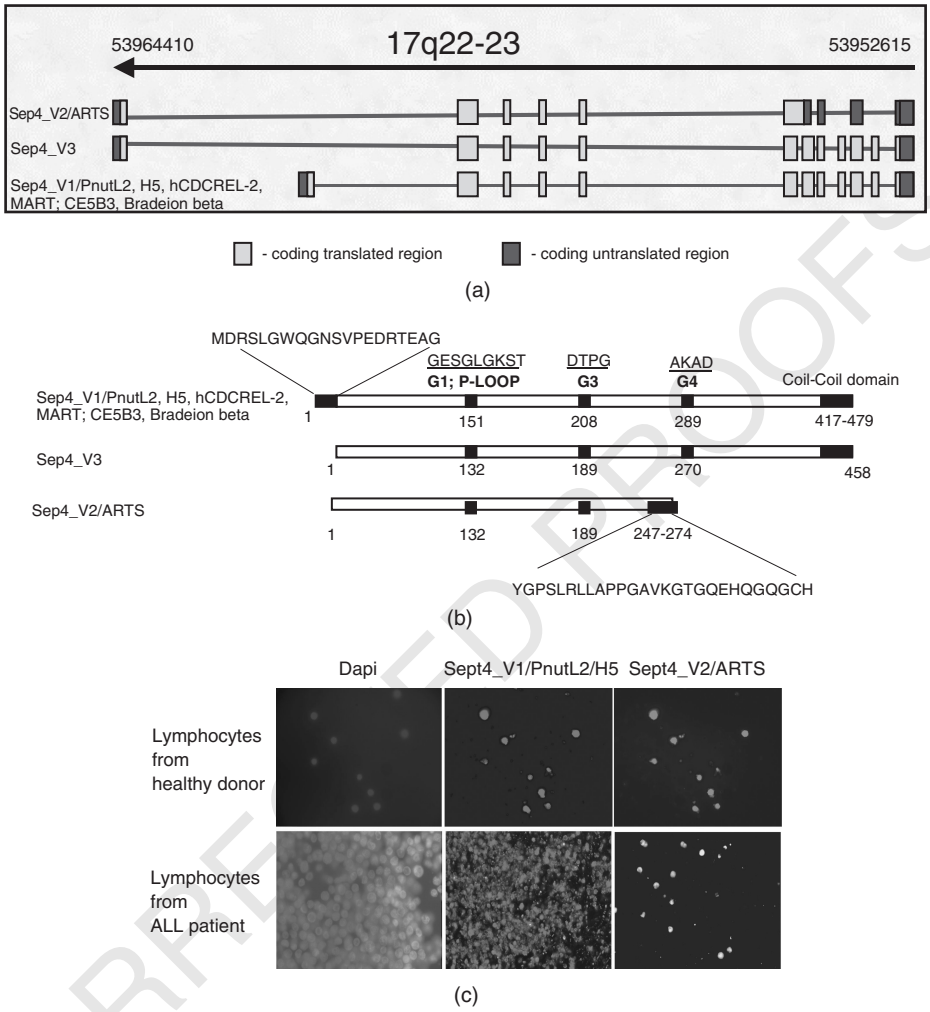


**Figure 13.1** (a) Schematic illustration of the different splice variants of Septin 4. The boxes indicate transcribed regions. *Sep4_V3 isoform awaits experimental validation. Current data support the existence of only two distinct isoforms for the human *Sept 4* gene. (b) Alignment of the different human Septin 4 isoform sequences. Location of the conserved G1 (P-loop) G3, G4 and coil–coil motifs are denoted by the hedged boxes. Unique sequences are marked as black boxes. (c). Sept4_V2/ARTS is the only human Septin4 isoform shown to directly induce and promote apoptosis. Sept4_V2/ARTS functions as a tumour suppressor protein. Immunofluorescence staining presents lymphocytes isolated from healthy donor (upper panel) and from acute lymphoblastic leukemia (ALL) patient (lower panel). Staining with Dapi, showing nuclei of cells (blue), the non-apoptotic isoform of Sept4; Sept4_V1/PnutL2/H5 (red), and pro-apoptotic protein Sept4_V2/ARTS (green). Whereas all lymphocytes in healthy control contain similar levels of ARTS and H5 (upper panel), the sample from leukemia patient exhibits loss of ARTS staining in all tumour lymphoblasts (only remaining normal cells are stained), indicating selective loss of the pro-apoptotic protein Sept4_V2/ARTS in leukemia patients (Adopted from Elhasid *et al*., 2004)

and Omi/HtrA2 (Du *et al*., 2000; Verhagen *et al*., 2000; Verhagen *et al*., 2002; Hegde *et al*., 2002). Yet, SEPT4_i2 exhibits a unique mode of action in activating caspases through antagonizing IAPs; SEPT4_i2 lacks any recognizable IBM (IAP-Binding-Motif), a short sequence that is necessary for IAP-binding and inhibition and that is conserved amongst all other known IAP-antagonists (Shi, 2002; Huang *et al*., 2001; Tittel and Steller, 2000; Vaux and Silke, 2003). Instead, the binding to XIAP requires the unique C-terminus of SEPT4_i2, same stretch of 27 amino acids which became exonic sequences only in the SEPT4_i2 isoform as a result of intron retention phenomenon. Consistent with this idea, deletion of the C-terminus of SEPT4_i2 results in loss of XIAP-binding (Gottfried *et al*., 2004; Larisch *et al*., 2000). SEPT4_i2 is the only protein shown to function as an IAP-antagonist *in vivo*; as increased XIAP levels were found in sperm from *Sept4* deficient mice (Kissel *et al*., 2005).

*SEPT4_i2 functions as a tumour suppressor protein*

Studies both in human patients and in mice have shown that SEPT4_i2 plays an important role in cancer, particularly in haematopoietic cancers, leukemia and lymphoma. Expression of SEPT4_i2 is lost in all lymphoblasts of more than 70 % of childhood acute lymphoblastic leukemia (ALL) patients. The loss of SEPT4_i2 is specific, as the related non-apoptotic Septin protein H5, bearing 83 % identity to SEPT4_i2, is unaffected (Elhasid *et al*., 2004). During remission, SEPT4_i2 expression is detected again in almost all patients. Two leukemic cell lines, ALL-1 and HL-60 lacking SEPT4_i2, were resistant to apoptotic induction by cytosine arabinoside. Transfection of *SEPT4_v2* into these cells restored their ability to undergo apoptosis in response to this chemotherapeutic agent (Elhasid *et al*., 2004). Moreover, a large percentage of the *Sept4* deficient mice exhibit extremely large spleens, and develop spontaneous haematopoietic malignancies (Steller, H. personal communication, unpublished results).

Altogether, it seems that a combination of serendipity along with rare genetic and biochemical events have turned SEPT4_i2 into an exceptional protein with unusual features both as a septin family member and as an IAP-antagonist protein. First, the rare intron retention phenomenon occurring in the *SEPT4* gene, which generates a novel stretch of amino acids not found in any other Septin or known protein. Second, this unique sequence which does not share any resemblance to other known IBM (IAPs binding motif) bears the ability to bind and antagonize IAPs, releasing caspases and leading to apoptotic death of the cell.

## DISCUSSION

Although generation of large number of splice variants is characteristic of the septin family of genes (see Chapter 7), only one splice variant of the *SEPT4* gene, *SEPT4_v2*/SEPT4_i2 is currently known to directly promote apoptosis. Nevertheless, the association of several septins with two pathological phenomenon, cancer

and neurodegenerative diseases may indicates that other Septins may be involved, at least indirectly, in apoptosis. But at present direct evidence for the role of septins in apoptosis in these, or other conditions, is lacking. Therefore, within the septin family, SEPT4_i2 could have a unique function that may have arisen as an evolutionary 'accident'. Through coincidental retention of an intron, a novel open reading frame (ORF) was generated at the *SEPT 4* locus that resulted in addition of a unique stretch of 27aa to the SEPT4_i2 isoform. This unique SEPT4_i2 sequence either had or evolved the capacity to bind IAPs, thereby endowing SEPT4_i2 with the ability to activate caspases and cell death (Gottfried *et al*., 2004; Larisch *et al*., 2000; Larisch-Bloch *et al*., 2000). The other *SEPT4* splice variants, SEPT4_i1 which share 83 % sequence homology with SEPT4_i2, cannot bind XIAP and promote apoptosis (Gottfried *et al*., 2004; Elhasid *et al*., 2004; Larisch *et al*., 2000). Moreover, loss of SEPT4_i2 in ALL patients is specific and related to its pro-apoptotic function, as levels of the H5 non-apoptotic Septin in these samples remained unaffected (Elhasid *et al*., 2004). All this data indicates that the unique C′-terminus of SEPT4_i2, which is not shared by any other Septin, is critical for its pro-apoptotic and tumour suppressor function. Does the P-loop GTPase domain, septins most conserved and characteristic sequence which is also present in SEPT4_i2, contribute to its pro-apoptotic function? First, SEPT4_i2 contains a non-typical GTP-binding domain which does not include the G4 motif (Figure 13.1b). One might argue that this lack of G4 motif might contribute in any way to its pro-apoptotic function. We therefore conclude that the P-loop of SEPT4_i2 may be necessary, but not sufficient for the induction of apoptosis. Furthermore, Lee *et al*. analysed the DNA sequences encoding the P-loop domains of SEPT4_i2 and Nod1 proteins in the tissues of colorectal carcinomas, gastric carcinomas, non-small cell lung cancers, and hepatocellular carcinomas and compared them to the P-loop sequence of normal cells originating from the same patients. The data analysis indicated that mutational events in both the P-loop domain of SEPT4_i2 and Nod1 genes do not contribute to the development of these cancers (Lee *et al*., 2006; Lee *et al*., 2006a). These studies support our model that the P-loop GTPase domain is not directly involved in apoptotic function, but perhaps plays a regulatory role for SEPT4_i2. The pro-apoptotic function of SEPT4_i2 is highly correlated with its function as a tumour suppressor protein. Expression of SEPT4_i2 is lost in all lymphoblasts of more than 70 % of childhood ALL patients (Elhasid *et al*., 2004). A screen for proteins interacting with Kaposin A, a human herpes virus involved in Kaposi Sarcoma, and other types of cancers, revealed possible functional interaction of Kaposin A protein with SEPT4_i2. The authors suggest that expression of Kaposin A protein could inhibit the apoptotic effect of SEPT4_i2 leading to reduced apoptosis and transformation of infected cells to cancer cells (Lin *et al*., 2007).

Most Septins are localized at the cytosol although nuclear localization is seen with some. In contrast SEPT4_i2 localizes to mitochondria in healthy cells. Another splice variant of the *SEPT 4* gene, M-Septin, is also reported to be localized in mitochondria of developing mouse neurons. M-Septin was not shown

to be involved in apoptosis (Takahashi *et al*., 2003). Therefore, it seems the localization of Septins in mitochondria per se does not necessarily indicate an apoptotic function. In the case of SEPT4_i2, its mitochondrial confinement serves as a compartmentalization mechanism used to prevent its interaction with IAPs which reside in the cytosol, interaction that could induce unwanted apoptosis via a C′-terminus unique stretch of 27aa which is not present in any other Septin or any other yet known protein.

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## Further Reading

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**Queries in Chapter 13**

Q1. Please confirm if the abbreviation GTP needs to be spelt out. If yes, please provide the expansion.