Biology and insights into the role of cohesin protease separase in human malignancies

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ABSTRACT

Separase, an enzyme that resolves sister chromatid cohesion during the metaphase-to-anaphase transition, plays a pivotal role in chromosomal segregation and cell division. Separase protein, encoded by the extra spindle pole bodies like 1 (ESPL1) gene, is overexpressed in numerous human cancers including breast, bone, brain, and prostate. Separase is oncogenic, and its overexpression is sufficient to induce mammary tumours in mice. Either acute or chronic overexpression of separase in mouse mammary glands leads to aneuploidy and tumorigenesis, and inhibition of separase enzymatic activity decreases the growth of human breast tumour xenografts in mice. This review focuses on the biology of and insights into the molecular mechanisms of separase as an oncogene, and its significance and implications for human cancers.

Key words: separase, ESPL1, sister chromatid cohesion and separation, cohesin, cell division, oncogene.

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I. INTRODUCTION

Separase is an enzyme that resolves chromosomal cohesion during mitosis. It was first identified in fungi, where the loss of separase function resulted in accumulation of extra spindle pole bodies and polyploid nuclei (Baum et al., 1988; Uzawa et al., 1990; May et al., 1992). Later, separase was found to be essential for sister-chromatid separation by cleaving cohesin subunit sister chromatid cohesion protein 1 (Scc1)/mitotic chromosome determinant

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(Mcl1)/radiation-sensitive mutant 21 (Rad21) (Uhlmann, Lottspeich & Nasmyth, 1999; Waizenegger et al., 2000). Separase is a cysteine protease in the CD clan, with a catalytic domain structure similar to that of caspas. All bonds known to be hydrolysed by this endopeptidase have arginine in P1 and an acidic residue in P4. P6 is often occupied by an acidic residue or by a hydroxyl-amino-acid residue, the phosphorylation of which enhances cleavage. The C-terminus of separase, which contains the proteolytic active site, is conserved from yeast to humans. In addition to its canonical role in sister-chromatid separation, separase is involved in other functions, such as centrosome addition to its canonical role in sister-chromatid separation, and regulation. Also, we discuss the role of separase in human cancers and its potential as an emerging target for cancer therapy.

II. MOLECULAR CHARACTERISTICS OF SEPARASE

Separase exists in all eukaryotes, ranging in size from 140 to 240 kDa. The regulatory domains reside on the N-terminus, and the catalytic domain is on the C-terminus. Bioinformatic analysis and electron microscopy indicate that separase proteins generally are ordered, globular proteins, with only a few disordered regions (Viadiu et al., 1996). The region 1380–1397 aa of human separase is homologous to the N-terminus of yeast Cdc6 that interacts with yeast Cdk1 (Gorr et al., 2005). Similar to the Cdk1 inhibitor domain of yeast Cdc6, the domain from 1380 to 1397 aa also binds to Cdk1/cyclin B. Moreover, this binding mutually deactivates both separase and Cdk1/cyclin B (Gorr et al., 2005).

Unlike separase in lower eukaryotes, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, separase in higher eukaryotes is auto-catalysed at the transition of metaphase to anaphase when the enzyme is activated. There are three adjacent auto-cleavage sites (EIMR1486, EILR1306, EILLR1335) in the hinge region of human separase that are conserved among vertebrates. However, auto-cleavage normally occurs at EILR1306 and EILLR1335 in cervical cancer line HeLa cells. EIMR1486 is not used unless EILR1306 is mutated (Waizenegger et al., 2002). The auto-cleaved fragments remain associated with each other for the separase molecule to be functional (Waizenegger et al., 2002; Zou et al., 2002). Mutation on separase auto-cleavage sites not only prevents auto-cleavage but also interferes with mitosis entry and progression (Papi et al., 2005), suggesting that separase auto-cleavage itself or the cleaved fragments play an important role in cell-cycle progression.
The separase protease domain (SPD) is located at the conserved C-terminus. The SPD is comprised of the pseudo protease domain (PPD) and active protease domain (APD), of which only the latter has protease activity (Viadiu et al., 2005; Lin, Luo & Yu, 2016). Although both the PPD and APD were reported to be caspase-like, only the APD has sequence and structural similarities to caspase (Winter et al., 2015; Lin et al., 2016). The catalytic site of separase contains two invariant residues, a histidine (H$^{2003}$) and a cysteine (C$^{2029}$), surrounded by regions typically found in cysteine proteases of the CD clan (Uhlmann et al., 2000). Homology modelling using caspase 3 as a template predicts that the caspase-like domain of C. elegans separase folds in a similar way to the functional heterodimer (p10 plus p20 subunits) of caspase (Winter et al., 2015).

The structure of full-length separase has not been solved. Electron microscopic images of negatively stained human separase–securin complex show that the complex has an elongated whale shape with securin binding to the N-terminus of the separase (Viadiu et al., 2005). The crystal structure of the separase protease domain from the thermophilic fungus Chaetomium thermophilum has recently been worked out (Lin et al., 2016), revealing how separase recognizes its substrate and how polo-like kinase 1 (Plk1)-phosphorylated cohesin Rad21 enhances cleavage. However, although homolog modelling reveals that the SPD structures of human separase and C. thermophilum separase are similar (Lin et al., 2016), the crystal structure of human separase remains to be resolved.

III. CELLULAR LOCALIZATION OF SEPARASE

The spatial localization of separase varies with the progression of the cell cycle. In interphase cells, separase is localized in the cytoplasm, but during mitosis it accumulates in the nucleus and associates with chromosomes (Kumada et al., 1998; Sun, Yu & Zou, 2006). Yeast has a closed mitosis, and how these sites compare with cohesin-binding sites is not known. Unlike normal cells, in tumour cells, separase is found to be localized to the interphase nuclei (Meyer et al., 2009). The role of separase in interphase nuclei is not fully known. A recent study (McAleenan et al., 2013) indicated that...
counterparts (Zou et al., 1999). Nonetheless, the function of securin in regulating separase activity is evolutionarily conserved from yeast to human. Results from biophysical analysis suggest that securin is unstructured in solution and behaves as an extended polypeptide (Sanchez-Puig, Veprintsev & Fersht, 2005). Securin has both positive and negative roles in regulating separase as separase-specific chaperone and inhibitor. It increases the solubility of separase by binding co-translationally via ribosome-nascent chain complex (RNC), whereas most separase in securin-null cells is insoluble (Hellmuth et al., 2015a), suggesting that securin functions as a chaperone to help proper folding of separase. Although securin has been reported to play a role in transcriptional regulation (Bernal et al., 2002; Hamid & Kakar, 2004; Tong & Eigler, 2009), recent studies indicate that securin has no effect on transcription, mRNA stability, or translation efficiency of the ESPL1 gene that encodes for separase protein (Hellmuth et al., 2015a).

Securin inhibits separase activity until the onset of anaphase. In budding yeast, securin binds strongly to the N-terminus but only associates weakly with the C-terminus of separase. Deletion of 155 aa of the N-terminus of separase significantly reduces the association of securin with separase (Hornig et al., 2002). Without securin, the first 155 aa N-terminal fragment of separase interacts with the last 400 aa C-terminal fragment that contains the protease domain of separase. Co-expression of securin disrupts the direct interaction between the N- and C-termini of separase (Hornig et al., 2002). Securin contains a conserved motif (D/E)xExxxxx, which matches the separase cleavage consensus at P6–P2. When this conserved motif is mutated into (D/E)xExxRD, securin can be cleaved by separase (Nagao & Yanagida, 2006; Lin et al., 2016). Researchers have speculated that this conserved motif of securin plays a role in physically blocking the enzymatic active site of separase by preventing the accession of substrates and their subsequent cleavage (Nagao & Yanagida, 2006; Lin et al., 2016).

Separase activity is also inhibited by phosphorylation-dependent Cdk1/cyclin B binding (Ciosk et al., 1998; Zou et al., 1999; Stemmann et al., 2001; Gorr et al., 2005; Holland & Taylor, 2006). After securin is degraded, separase forms a complex with Cdk1/cyclin B1 and Cdk1-associated subunit cyclin-dependent kinases regulatory subunit 2 (Cks2). There are two Cdk1/cyclin B-binding determinants in human separase; (i) phosphorylation at S1126 and T1346 residues, and (ii) sequence-specific binding motif 1380–1397 aa, similar to a known Cdk1-inhibiting motif in yeast Cdc6 (Gorr et al., 2005). Mutation of S1126, T1346 or Cdk1/cyclin B-binding motif on separase abolishes the interaction between separase and Cdk1/cyclin B (Gorr et al., 2005). For the formation of a separase-Cdk1/cyclin B complex, phosphorylation on separase, but not the kinase activity of Cdk1/cyclin B1, is essential. Cdk1/cyclin B1 binds to the middle part of separase (Gorr et al., 2005), which is a different location from that where securin binds to separase (N- and possible C-terminus) (Hornig et al., 2002; Jager et al., 2004; Viadiu et al., 2005). Possibly, a specific configuration is induced when separase binds to securin or Cdk1/cyclin B, which explains the mutual exclusion of securin and Cdk1/cyclin B-forming complexes with separase simultaneously.

Although phosphorylation of separase on S1126 facilitates separase conformation change for the binding of Cdk1/cyclin B, it reduces the solubility of separase (Hellmuth et al., 2015a). It is plausible that once separase is phosphorylated and conformation is changed, association with Cdk1/cyclin B is required for its solubility. This possibility is supported by observations that, compared to separase WT, the phosphorylation mutant separase S1126A

**Fig. 2.** Immunofluorescence microscopy of separase. HeLa cells were cytopspun to slides, fixed with paraformaldehyde, and permeabilized with Triton X-100. The cells were stained with lamin B1 (green) and separase (red) antibodies. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 5 μm.

### IV. REGULATION OF SEPARASE ACTIVITY

Separase activity is regulated by its inhibitory chaperones: securin and Cdk1/cyclin B. Human securin is encoded by pituitary tumour-transforming gene 1 (PTTG1). Securins from vertebrates share extensive sequence similarity with each other but have very little similarity to their yeast counterparts (Zou et al., 1999). Nonetheless, the function of securin in regulating separase activity is evolutionarily conserved from yeast to human. Results from biophysical analysis suggest that securin is unstructured in solution and behaves as an extended polypeptide (Sanchez-Puig, Veprintsev & Fersht, 2005). Securin has both positive and negative roles in regulating separase as separase-specific chaperone and inhibitor. It increases the solubility of separase by binding co-translationally via ribosome-nascent chain complex (RNC), whereas most separase in securin-null cells is insoluble (Hellmuth et al., 2015a), suggesting that securin functions as a chaperone to help proper folding of separase. Although securin has been reported to play a role in transcriptional regulation (Bernal et al., 2002; Hamid & Kakar, 2004; Tong & Eigler, 2009), recent studies indicate that securin has no effect on transcription, mRNA stability, or translation efficiency of the ESPL1 gene that encodes for separase protein (Hellmuth et al., 2015a).

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increases solubility, whereas the separase mutant lacking the Cdk1/cyclin B-binding motif decreases solubility (Hellmuth et al., 2015a).

Separase and Cdk1/cyclin B1 binding mutually inhibit the proteolytic activity of separase and the kinase activity of Cdk1. Mutations on S1126 and T1346 residues on separase do not inhibit separin degradation but reduce the ability of separase to inactivate Cdk1/cyclin B1 and delay/prevent mitotic exit (Gorr et al., 2005), implying that formation of the separase and Cdk1/cyclin B complex facilitates mitotic exit by concomitantly attenuating the activity of separase and Cdk1/cyclin B.

Both separin and cyclin B contain the destruction box and KEN box in their N-termini, are substrates of the anaphase-promoting complex/cyclosome (APC/C), and degrade at the metaphase and anaphase transition with the same kinetics (Haging et al., 2002). The C-termini of separin and cyclin B are responsible for their physical interactions and inhibition of separase enzymatic activity (Gorr et al., 2005; Han & Poon, 2013), suggesting that the N-terminus of the inhibitor in either the separase–separin complex or the separase–Cdk1/cyclin B complex can be freely accessed by APC/C and degraded in order to activate separase. The coexistence of both complexes is likely a failsafe mechanism to regulate separase if one of the inhibitors is lost; the fact that separin null cells have a relatively normal cell cycle supports this notion.

Recent studies indicate that phosphorylation of separase is required to make a configuration switch in order to bind to Cdk1/cyclin B (Hellmuth et al., 2015b). The isomerase Pin1, a 17 kDa PPIase of the parvin subfamily, is responsible for this switch. Its N-terminal WW domain and C-terminal PPIase domain can bind to phosphorylated Ser/Thr-Pro (pS/T-P) motifs but with a different peptide-binding specificity in separase. The WW domain recruits Pin1 to separase by binding to pS1153P and enables the PPIase domain to engage pS1126P. Both depletion of Pin1 or chemical inhibition of Pin1’s PPIase activity by epigallocatechin-3-gallate (EGCG) can inhibit cyclin B1 binding to separase. As a result, activated separase causes premature loss of sister-chromatid cohesion (Hellmuth et al., 2015b).

Separin and cyclin B1 cannot bind to separase simultaneously; the same is true for separin and Pin1. However, Pin1, cyclin B1, and separase are found in the same complex. Separase’s association with separin is in the trans isomer. After separase is phosphorylated at S1126 and separin is degraded, Pin1 switches separase from trans to cis isomer, which exposes its CLD (1342–1404 aa on human separase; Fig. 1) for cyclin B1 binding. Once separase becomes a cis isomer, its ability to re-associate with separin is significantly reduced, thereby protecting it from separin-mediated inhibition (Hellmuth et al., 2015b).

Although Cdk1/cyclin B1 can bind to separase once separin is degraded, the peak of the separase-Cdk1/cyclin B1 complex forms after cohesin Rad21 is cleaved. Compared to the WT cyclin B1, non-phosphorylatable cyclin B1 has much higher affinity of binding to separase (Hellmuth et al., 2015b). Because cyclin B1 is phosphorylated in several sites in early mitosis (Toyoshima-Morimoto et al., 2001; Jackman et al., 2003; Daub et al., 2008) and some of these sites are dephosphorylated during mitotic exit, it is plausible that separase binding to dephosphorylated cyclin B1 is a mechanism to stop separase activity once sister chromatids segregate. Moreover, the cis isomer of separase is less stable than is the trans isomer. It is possible that the trans to cis isomerization of Pro1127 in separase limits the half-life of its proteolytic activity, which ensures timely activity of separase at the onset of anaphase to cleave Rad21 and to be deactivated after the segregation of sister chromatids. Deactivation of separase might occur via conformation change because the level of separase protein is relatively constant before and after deactivation (Hellmuth et al., 2015b). In this context, how separase activity is switched off remains to be determined.

V. SUBSTRATES OF SEPARASE

As an endopeptidase, one function of separase is to cleave protein substrates. However, only a few proteins have been identified as separase substrates. In yeast, cohesin mitosis-specific subunit Scc1/Mcd1, cohesin meiosis-specific subunit Rec8, and kinetochore and spindle protein Slk19 are the only known substrates of separase (Uhlmann et al., 1999; Buonomo et al., 2000; Sullivan, Lehane & Uhlmann, 2001), whereas Rad21, Rec8, and pericentrin (PCNT)/kendrin are the substrates of separase in mammalian cells (Lee & Rhee, 2012; Matsuo et al., 2012; Kim, Lee & Rhee, 2015). The core motif cleaved by separase in yeast is (D/E)xR, and that in vertebrates is ExxR. Separase cleaves the peptidyl bond after arginine residues of the core motif. Although approximately 20% of the proteins in yeast contain the core motif, only three (Scc1, Rec8 and Slk19) have been confirmed as separase substrates (Sullivan et al., 2004). Interestingly, human separase cannot cleave yeast Rad21, and vice versa (Waizenegger et al., 2002). It is not very clear what factors determine the specificity of separase’s cleavage of its substrates. According to one study, one of the determining factors is the adjacent amino acid residues before P1 arginine (R) and P4 acidic amino acid residue aspartic acid/glutamic acid (D/E) (Sullivan et al., 2004; Winter et al., 2015). For example, systematic mutagenesis indicates that the cleavage consensus motif of separase in budding yeast is (not F/K/R/W/Y)(A/C/F/H/I/L/M/P/V/W/Y)D/E|x(A/G/S/V)R (Sullivan et al., 2004). In other words, in order to be cleaved by separase, the amino acid residue at P6 should not be (F/K/R/W/Y), (A/C/F/H/I/L/M/P/V/W/Y) should be at P5, D or E is required at P4, any amino acid can be at P3, (A/G/S/V) should be at P2, and R must be at P1.

Another factor is the interaction of separase and the part of the substrate protein other than the cleavage motif, which may facilitate the fit between the catalytic site of separase and the cleavage motif of the substrate. In
addition, post-translation modifications may also regulate the efficiency of cleavage. For instance, phosphorylation of both Rad21 and pericentrin by Plk1 yields better substrates for separase (Haul et al., 2005; Kim et al., 2015; Monen et al., 2015). Because separase appears to be involved in numerous functions in the cell cycle, more substrates and interactors likely will be identified.

VI. FUNCTION OF SEPARASE

(1) Chromosome cycle

The canonical function of separase is to cleave cohesin at the onset of anaphase to release sister-chromatid cohesion. In mitotic cells, DNA is replicated at the S phase and the sister chromatids are tethered together by cohesin complexes. In higher eukaryotes, cohesins are removed in two steps. Cohesins on chromosome arms are released via a separase-independent mechanism at prophase. Those on centromeres and remaining on chromosome arms are removed after separase is activated and Rad21 is cleaved by separase at the transition between the metaphase and anaphase. Sister chromatids are pulled to the opposite poles by spindle microtubules to complete their separation. In meiosis, cohesin complexes are removed in two steps, as occurs in mitosis. During meiotic prophase I, chromosomes undergo alignment, pairing, synopsis, and recombination, all of which are required for homologous chromosomes to be tethered to the meiotic spindles in pairs. Cohesin not only tethers sister chromatids but also the part of homolog chromosomes where homologous recombination occurs (Rankin, 2015; Sansam & Pezza, 2015). At the anaphase of meiosis I, cohesin complexes are removed from the chromosome arms in a separase-independent manner (Severson & Meyer, 2014), allowing separation of homologs (disjunction). Shugoshin 1 (Sgo1) and PP2A protect the cohesins on centromeres from cleavage by separase (Kitajima et al., 2006; Riedel et al., 2006; Chambon et al., 2013; Rattani et al., 2013). At anaphase of meiosis II, centromeric cohesins that are no longer protected by Sgo1 and PP2A are cleaved by separase, resulting in the separation of sister chromatids, ultimately leading to the generation of four haploid gametes from each meiotic cell.

(2) Centrosome cycle

Similar to its proteolytic cleavage of cohesin Rad21 during the separation of sister chromatids in the metaphase-to-anaphase transition, separase activity is also required in the disengagement of centrioles. Two separase inhibitors, cyclin B1 and securin, can inhibit centriole disengagement (Tsou & Stearns, 2006). Depletion of separase also blocks centriole disengagement (Thein et al., 2007). Cohesin has been identified in centrosomes. Cleavage of cohesin Rad21 (Nakamura et al., 2009; Tsou et al., 2009; Schockel et al., 2011) and centrosomal protein pericentrin/kendrin (Lee & Rhee, 2012; Matsumo et al., 2012) by separase in centrosomes further confirms the requirement for separase in centriole disengagement. Although the exact timing and the regulatory network governing centriole disengagement remain to be fully defined, the consensus is that centriole disengagement occurs during the late mitosis/early G1 phase (Tsou et al., 2009; Beauchene et al., 2010; Schockel et al., 2011), which occurs after the separation of sister chromatids. After securin and cyclin B1 are degraded, what prevents the activated separase from disengaging centrioles is not fully understood.

(3) DNA damage repair

Cohesin is required for DNA double-strand break (DSB) damage repair, which is conserved from yeast to humans (Sjogren & Nasmyth, 2001; Sonoda et al., 2001; Kim et al., 2002; Atienza et al., 2005). In response to post-replicative DNA DSB, cohesin is recruited to reinforce cohesion of the sister chromatids and is accumulated at the break to enforce physical proximity to facilitate homologous recombination-mediated repair (Unal et al., 2004; Strom et al., 2007; Kim et al., 2010). In fission yeast, separase-mediated Rad21 cleavage is required during the repair of DNA damage caused by ultraviolet (UV) irradiation as well as X-ray and γ-ray irradiation (Nagao et al., 2004). In budding yeast, resection of the break is required to generate longer 3’ single-strand tails to use sister chromatids as templates for homology-dependent repair. To facilitate this event, separase is essential to remove the cohesin complexes around the break, which are loaded during DNA replication in the S phase and after DNA damage. Expression of separase-resistant Rad21 or mutant separase reduces the DSB resection and damage repair (McAleenan et al., 2013). The cohesin complexes used for DNA damage-induced cohesion are different from those used for generation of sister-chromatid cohesion in the S phase. In yeast, Rad21 in the damage-induced cohesion is phosphorylated and acetylated (Heidinger-Pauli et al., 2008; Heidinger-Pauli, Unal & Koshland, 2009). After completion of the DSB repair, Rad21 cleavage by separase dissolves the extra cohesion induced by DNA damage (Adachi et al., 2008). How separase activity is regulated during DNA damage repair remains unclear, but researchers speculate that it should be controlled tightly and locally.

(4) Membrane trafficking

In many organisms, degranulation occurs by releasing the cargo proteins in cortical granules following fertilization to modify the extracellular covering of the zygote. In Caenorhabditis elegans, degranulation has been found in a wave starting in the vicinity of the meiotic spindle during anaphase I. Separase is required for this process. Separase is co-localized cortically to filamentous structures in prometaphase I upon maturation of the oocyte. After fertilization, separase is relocated from these structures to cortical granules by the time anaphase I occurs. Knockdown of separase using RNAi inhibits degranulation.
in addition to causing abnormal chromosomal segregation (Bembenek et al., 2007). Several mutations of separase with little effect on chromosomal segregation reduce its localization to cortical granules (Bembenek et al., 2007; Richie et al., 2011), suggesting that the functions of separase in membrane trafficking and chromosomal segregation are independent.

During mitosis of the C. elegans embryo, separase localizes to the ingressing furrow and midbody during cytokinesis. Loss of separase function during the early mitotic divisions causes accumulation of RAB-11-positive vesicles at the cleavage furrow and midbody and results in cytokinesis failure, which resembles the depletion of vesicle fusion machinery. This suggests that separase is required for cytokinesis by regulating the incorporation of the vesicles essential for plasma membrane assembly at the cleavage furrow and midbody (Bembenek, White & Zheng, 2010). The phenotypes of cortical granule exocytosis and cytokinesis caused by mutant separase can be suppressed by a mutation in the protein phosphatase 5 (pph-5) gene (Richie et al., 2011), implying that function of separase in vesicle trafficking is regulated by phosphorylation/dephosphorylation.

In mammalian cells, both securin and separase associate with membranes. Knockdown of either protein causes swelling of the trans-Golgi network and endocytic vesicles, diminishing constitutive protein secretion and impairing receptor recycling and degradation (Bacac et al., 2011). Further studies indicate that depletion of securin or separase causes defective acidification of early endosomes and increases membrane recruitment of vacuolar (V)-ATPase complexes (Bacac et al., 2011), implying that securin and separase modulate membrane traffic and protein secretion by regulating V-ATPase assembly and function.

VII. ROLE OF SEPARASE IN HUMAN CANCER

(1) Separase is an oncogene

Numerous recent studies have established separase as an oncogene, which is overexpressed in many human cancers of breast, bone, brain, and prostate (Pati, 2008; Zhang et al., 2008; Meyer et al., 2009; Mukherjee et al., 2014a,b) (Fig. 3). Among these tumours, overexpression of separase in human breast cancer tumours has been well characterized. Compared to matched, normal breast tissue, more than 60% of breast tumours overexpress separase (Zhang et al., 2008). Approximately a quarter of the sepa-rase-overexpressing tumours are luminal-B subtype with altered p53 function, a category of breast tumours that often develop endocrine resistance (Carey et al., 2006; Dawood et al., 2011). Approximately 50% of triple-negative breast cancer (TNBC) tumours have either high (22%) or intermediate (28%) levels of separase expression in their non-proliferating cells. Analysis of the cancer genome atlas (TCGA) data set (Cancer Genome Atlas Network, 2012) indicates that more than 53% of breast tumours overexpress ESPL1 transcript (Mukherjee et al., 2014b). With the exception of luminal-A tumours, all major subtypes have an average twofold increase in the ESPL1 transcript, corroborating immunostaining and Western blot data (Zhang et al., 2008; Meyer et al., 2009). Meta-analysis also indicates a strong positive correlation between separase mRNA expression and tumour grade, as well as a strong negative correlation with disease-free and overall survival (Meyer et al., 2009; Mukherjee et al., 2014b).

According to an integrated RNA-seq-derived gene expression database, 96% of breast and 84% of prostate tumours overexpress ESPL1 transcripts. Compared to normal tissue, ESPL1 transcripts appear to be overexpressed in many human tumour types, with the exception of kidney tumours (Fig. 3). The average mutation frequency on the ESPL1 coding region in various cancer types is insignificant, at about 1.3% (http://www.tumorportal.org/; Table 1), and the mutation sites are found to be scattered on the entire coding region (Table 2). Although the mutation frequency of ESPL1 seems low and gain-of-function caused by mutation has not been reported, overexpression of separase might result from the deregulation of other cell processes, such as transcription, translation, and degradation. The putative progesterone responsive element (PRE), estrogen responsive element (ERE), p53 transcriptional activation element (p53TAE), and p53 transcriptional repressor elements (p53TRE) on the ESPL1 promoter region have been identified (Pati et al., 2004; Pati, 2008). Deregulation of such transcription factors could affect expression of separase.

(2) Separase overexpression promotes aneuploidy and genetic heterogeneity

In a tetracycline-inducible system (Zhang et al., 2008) and mammary-gland-specific, separase-transgenic mice (Mukherjee et al., 2014b), conditional overexpression of separase in mouse mammary glands induced not only aneuploidy but also mammary tumorigenesis. Induction of separase ex vivo into a clone of mouse mammary gland epithelial cells for a short period (3–5 days) resulted in varying levels of trisomies and monosomies and complex aneuploidies, suggesting genetic heterogeneity within the same population of cells (Zhang et al., 2008). Overexpression of separase resulted in premature separation of chromatids (PCS), lagging chromosomes, and anaphase bridges. Increased PCS and formation of anaphase bridges are indicators of chromosomal instability (CIN) and development of aneuploidy. In contrast to separase overexpression, homozygous deletion of the separase-encoding gene Espl results in early embryonic lethality in mice (Kumada et al., 2006; Wirth et al., 2006; Mukherjee et al., 2011) and zebrafish (Danio rerio) (Shepard et al., 2007). However, Espl heterozygote mice with significantly lower levels of separase protein have no apparent phenotype (Kumada et al., 2006; Wirth et al., 2006; Mukherjee et al., 2011). Using a hypomorphic separase mouse model, separase hypomorphs...
are genetically stable, have a cancer-free phenotype (Kumada et al., 2006; Wirth et al., 2006; Mukherjee et al., 2011), and live significantly longer compared to WT C57BL/6 littermate mice (Mukherjee et al., 2011). These studies suggest that attenuation of separase activity in separase-overexpressed cancer cells represents a new avenue to combat chromosomally unstable tumours.

(3) Transgenic mice overexpressing separase develop mammary tumours

To express separase specifically in mammary gland cells, a transgenic mouse model has been developed in which separase overexpression is under the control of a mouse mammary tumor virus (MMTV) promoter and induced by lactation (Mukherjee et al., 2014b). Transgenic mice expressing the MMTV-separase cDNA develop aneuploidy, with high-grade and highly heterogeneous mammary adenocarcinomas of both luminal and basal subtypes, with 80% penetrance at a latency of 12 months (Mukherjee et al., 2014b). The tumours induced by separase express various levels of luminal epithelial markers, including keratin-8 and E-cadherin, with abundant expression in well-differentiated tumour regions but greatly diminished or absent expression in poorly differentiated areas. Nests of carcinoma cells variably express basal/myoepithelial lineage markers (keratins-5, -14, vimentin), with populations of cells either positive or negative for ERα but all cells are negative for progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Aneuploid mammary glands and tumours from the MMTV-separase mice have significantly higher levels of DNA damage, as assessed by histone γH2AX foci and p53-binding protein 1 (53BP1) staining. Importantly, progressive loss of p53 expression is observed in premalignant mammary glands, and a near-complete loss is seen in mammary tumours, suggesting a role of separase in gene regulation. More than 40% of these tumours also show higher expression of cyclin D1 in their well-differentiated regions and loss of cyclin-dependent kinase (CDK) inhibitor p27

Fig. 3. RSEM mRNASEq expression profiles of separase gene ESPL1 in human cancers. Data from Firebrowse Gene Expression Viewer (http://firebrowse.org/viewGene.html?gene=espl1). Each box represents the range of separase mRNA expression of the disease cohort from first quartile to third quartile. The black line inside box is the median expression. The whiskers below and above each box indicate the lowest and highest expression, respectively. ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; COADREAD, colorectal adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, oesophageal carcinoma; GBM, glioblastoma multiforme; GBMLGG, glioma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIPAN, pan-kidney cohort (KICH + KIRC + KIRP); KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; STES, stomach and oesophageal carcinoma; TGCT, testicular germ cell tumours; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.
(CDKN1B). Loss of p27 has been associated with Tamoxifen resistance in ERα-positive human breast cancer (Cariou et al., 2000; Miller et al., 2008; Musgrove & Sutherland, 2009) and is also an independent marker for more invasive breast carcinomas in humans.

Similar to the tetraclyline (Tet-on)-induced separase-overexpressed cell lines and xenograft mouse model discussed above, overexpression of MMTV-separase also induces a number of CIN phenotypes, including appearance of micronuclei, multi-nucleated cells, chromosome bridges, aberrant numbers of centrosomes, and PCS. These findings suggest that hyperactive separase results in prematurely segregated chromosomes and lagging chromosomes at anaphase, which is indicative of chromosomal instability and development of aneuploidy. An interesting note is that two predominant populations of aneuploid cells, one with 37 and one with 45 modal chromosomes, were found in the MMTV-separase mammary epithelium. The cells with 37 chromosomes have loss of one copy of chromosome 2 and both copies of X, whereas the cells with 45 chromosomes have a Robertsonian translocation of chromosome 2 and trisomy for chromosomes 1, 3, 6, 11, 13, 15, and 18 (Mukherjee et al., 2014b).

### Table 1. Frequency of somatic mutations on ESPL1 in different types of cancers. The mutations include deletion, insertion, and substitution. Data from the catalogue of somatic mutations in cancer (COSMIC) database (V76) (cancer.sanger.ac.uk) (Forbes et al., 2015).

| Cancer type                        | Samples | Mutation (%) |
|------------------------------------|---------|--------------|
|                                    | Mutated | Tested       |
| Autonomic ganglia                  | 1       | 748          | 0.13         |
| Biliary tract                      | 4       | 329          | 1.22         |
| Bone                               | 3       | 509          | 0.59         |
| Breast                             | 11      | 1397         | 0.79         |
| Central nervous system             | 11      | 2163         | 0.51         |
| Cervix                             | 5       | 322          | 1.55         |
| Endometrium                        | 14      | 640          | 2.19         |
| Haematopoietic and lymphoid tissue | 11      | 2925         | 0.38         |

### (4) Gene mutations caused by separase overexpression

Potential consequences of chromosomal missegregation resulting from separase overexpression, including premature separation of chromatids, anaphase bridge, and DNA damage due to untimely removal of chromosomal cohesion, resulting in aneuploidy and tumorigenesis in animal models (Zhang et al., 2008; Mukherjee et al., 2014b), are summarized in Fig. 4. Studies have shown that chromosomal missegregation can result in both aneuploidy and DNA damage (Janssen et al., 2011)—tumorigenesis triggers that are typically considered to be independent phenomena. Chromosome missegregation errors in mammalian cells lead to DSBs, a result of lagging anaphase chromosomes trapped in the cleavage furrow during cytokinesis (Janssen et al., 2011; Holland & Cleveland, 2012). In addition to damage caused directly by the mitotic machinery, lagging chromosomes, including those that are missegregated, often form micronuclei, which over time also accumulate high levels of DNA damage, resulting in point mutations and chromosome fragmentations or shattering, a phenomenon termed chromothripsis (Liu et al., 2011; Magrangeres et al., 2011; Stephens et al., 2011; Crasta et al., 2012). The spectrum of mutations resulting from chromosome missegregation and the ensuing aneuploidy and DNA damage are not known. Additionally, it is not known how aneuploidy is tolerated, or how it may drive CIN, including genome rearrangements, translocations, and copy number changes that give rise to genetic variations, tumorigenesis, and tumour heterogeneity, the last of which poses a significant challenge to providing personalized cancer medicine (Burrell et al., 2013). It is plausible that mutations could arise from separase-induced chromosomal missegregation, and these mutations may play an important role for aneuploidy tolerance and tumour development, for an example by affecting the p53 pathway.

### (5) Separase inhibitors curtail the growth of human tumours

Modulating the activity of overexpressed separase to target chromosomal missegregation and to eliminate aneuploid cancer cells that are addicted to separase overexpression or sensitizing the cancer cells for further therapy could be a new strategy for cancer therapy. Separase is an attractive drug target for several reasons. First, it is a promotor of aneuploidy (Zhang et al., 2008; Mukherjee et al., 2014b) and is overexpressed in pan-human cancers including breast tumours, prostate tumours, osteosarcoma (Meyer et al., 2009), and glioblastoma (Mukherjee et al., 2014a). A quarter of separase-overexpressing human breast cancer tumour specimens are also luminal-B subtype, with altered p53 function, a category of breast tumours that often develop endocrine resistance with an aggressive disease phenotype and have no successful therapy (Carey et al., 2006; Dawood et al., 2011). Therefore, pharmacological attenuation of separase activity, constitutes a novel
Table 2. Mutations on ESPL1 are found in two or more tumor samples. The mutations include missense mutation and nonsense mutation (*). Somatic status indicates the information on whether the sample was reported to be confirmed somatic (confirmed), previously reported (reported), or variant of unknown origin (unknown). Data were from Catalogue of Somatic Mutations in Cancer (COSMIC) database (V76) (cancer.sanger.ac.uk) (Forbes et al., 2013).

| Mutation | AA       | CDS      | Sample name | Primary tissue | Histology       | Somatic status | Zygosity | PubMed Id |
|----------|----------|----------|-------------|----------------|-----------------|----------------|----------|-----------|
| p.I53V   | c.157A>G | HCC69T   | Liver       | Carcinoma      | Reported        | Heterozygous   | —        | —         |
| p.I53V   | c.157A>G | sussy-880T| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.R133H  | c.398G>A | 8065783  | Pancreas     | Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.R133H  | c.398G>A | 8064559  | Pancreas     | Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.A171S  | c.511G>T | pfq065T  | Stomach      | Carcinoma      | Confirmed        | Heterozygous   | —        | —         |
| p.A171G  | c.512G>G | SNUH_G39_S1| HL           | Haematopoietic | Confirmed        | Unknown        | —        | —         |
| p.E186*  | c.566G>T | LUAD-B00915| Lung        | Carcinoma      | Unknown         | Unknown        | —        | 22980975  |
| p.E186D  | c.558G>T | TCGA-AP-A059-01| Endometrium | Carcinoma | Unknown         | Heterozygous   | —        | —         |
| p.A57T   | c.1669G>A| TCQA-QG-A52Z-01| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.A57V   | c.1670C>T| TCQA-A6-2686-01| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.R596W  | c.1786C>T| TCQA-AA-3715-01| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.R596Q  | c.1787G>A| ESC-225T  | Oocytes      | Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.R72C   | c.2179C>T| YUWAND   | Skin         | Malignant melanoma | Confirmed       | Unknown        | —        | —         |
| p.R72C   | c.2179C>T| TCQA-EE-A2MR-06| Skin        | Malignant melanoma | Confirmed       | Unknown        | —        | —         |
| p.R72C   | c.2179C>T| TCQA-FR-A3YN-06| Skin        | Malignant melanoma | Confirmed       | Unknown        | —        | —         |
| p.R72C   | c.2179C>T| TCQA-FW-A3R3-06| Skin        | Malignant melanoma | Confirmed       | Unknown        | —        | —         |
| p.R76W   | c.2299C>T| MOLT-4    | HL           | Lymphoid neoplasm | Confirmed       | Heterozygous   | —        | 23856246  |
| p.R76W   | c.2299C>T| TCQA-B7-5816-01| Stomach     | Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.R76Q   | c.2300G>A| LUAD-YINHD | Lung         | Carcinoma      | Unknown         | Unknown        | —        | 22980975  |
| p.R72C   | c.2474C>T| TCQA-CG-475-01| Stomach     | Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.T82C   | c.3073C>T| TCQA-GF-A6C9-06| Skin        | Malignant melanoma | Confirmed       | Unknown        | —        | —         |
| p.R1025C | c.3074G>A| T96       | Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.S1049L | c.3146C>T| TCQA-D5-6928-01| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.R1025H | c.3074G>A| BD57T     | Biliary tract| Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.S1213T | c.3637T>A| LAU63     | Skin         | Malignant melanoma | Unknown         | Heterozygous   | —        | 22197931  |
| p.S1213F | c.3638C>T| OSCC-41-T | Skin         | Carcinoma      | Confirmed        | Heterozygous   | —        | 25303977  |
| p.P1303T | c.3907C>T| TCQA-GL-8500-01| Kidney      | Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.P1303L | c.3908C>T| 16        | Skin         | Malignant melanoma | Confirmed       | Unknown        | —        | 24438835  |
| p.G1357V | c.4070G>T| LUAD-CHTN- MAD06-00668| Lung  | Carcinoma | Unknown         | Unknown        | 22980975  |
| p.G137D  | c.4070G>A| ACINAR04   | Pancreas     | Carcinoma      | Confirmed        | Unknown        | —        | 24292923  |
| p.R1698C | c.5092C>T| TCQA-AA-3966-01| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | 22810696  |
| p.R1698H | c.5093G>A| LS174T    | Large intestine | Carcinoma | Confirmed        | Unknown        | —        | 24554717  |
| p.R1698H | c.5093G>A| LS180     | Large intestine | Carcinoma | Confirmed        | Unknown        | —        | 24755471  |
| p.L17118* &* | c.5121_5122delTG | T3021 | Large intestine | Carcinoma | Confirmed        | Unknown        | —        | 23344691  |
| p.L17118* &* | c.5121_5122delTG | T3021 | Large intestine | Carcinoma | Confirmed        | Unknown        | —        | 22810696  |
| p.V17121M | c.5161G>A| TCQA-EE-A5MR-01| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.V17121M | c.5161G>A| TCQA-EE-A2ID-06| Large intestine | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.I742L  | c.5225C>T| OSCC-GB_00410111| Upper aerodigestive tract | Carcinoma | Confirmed        | Unknown        | —        | —         |
| p.I742L  | c.5225C>T| 41T       | Upper aerodigestive tract | Carcinoma | Confirmed        | Unknown        | 24292195  |
| p.A1763T | c.5287G>A| TCGA-06-0122-01| Central nervous system | Glioma | Confirmed        | Unknown        | —        | —         |
| p.A1763T | c.5287G>A| TCGA-06-0122| Central nervous system | Glioma | Confirmed        | Unknown        | 23917401  |
| p.R1774* | c.5320C>T| TCQA-55-7724-01| Lung        | Carcinoma      | Confirmed        | Unknown        | —        | —         |
| p.R1774K | c.5320_5321C>G>AA | LUAD-NYU284 | Lung        | Carcinoma      | Unknown         | Unknown        | —        | 22980975  |
| p.R1780W | c.5338C>T| LS174T    | Large intestine | Carcinoma | Confirmed        | Unknown        | —        | 24554717  |
| p.R1780W | c.5338C>T| LS180     | Large intestine | Carcinoma | Confirmed        | Unknown        | —        | 24755471  |
| p.R1879H | c.5636G>A| BL4       | HL           | Lymphoid neoplasm | Confirmed       | Unknown        | —        | 23143595  |
| p.R1879H | c.5636G>A| tumor1490415| HL            | Lymphoid neoplasm | Confirmed       | Unknown        | —        | —         |
| p.R1966* | c.5896C>T| TCQA-AP-A056-01| Endometrium | Carcinoma | Reported        | Heterozygous   | —        | —         |
| p.R1966* | c.5896C>T| TCQA-EE-A29D-06| Skin        | Malignant melanoma | Confirmed       | Unknown        | —        | —         |
| p.A2052V | c.6155G>T| MOLT-4    | HL           | Lymphoid neoplasm | Confirmed       | Heterozygous   | —        | 23856246  |
| p.A2052V | c.6155G>T| BCM723T   | Liver        | Carcinoma      | Confirmed        | Unknown        | —        | —         |

HL, haematopoietic and lymphoid.
strategy to treat these tumours. Second, histopathologically, separase-overexpressing tumours are highly heterogeneous, showing features of both luminal and basal subtypes of breast cancer, with aggressive disease phenotype. Third, inhibitors that selectively inhibit separase enzymatic activity have shown great promise in preclinical studies (Zhang et al., 2014). Sepin-1, one of the separase inhibitors identified in a high throughput screening, inhibits the growth of separase-overexpressing human TNBC xenografts in mice in a separase-dependent manner (Zhang et al., 2014); in the same assay, Sepin-1 had no appreciable effect on TNBC tumours with low-separase expression, suggesting the specificity and efficacy of this compound in targeting tumours addicted to separase overexpression. Thus separase inhibitors are emerging as a new class of agents for cancer therapy. We anticipate that the status of separase expression of human tumours could be used as a new classification for personalized therapeutic intervention to treat human cancers with separase inhibitors.

VIII. CONCLUSIONS

(1) The canonical role of separase is to cleave cohesin Rad21 and facilitate the segregation of sister chromatids. This function is executed by the separase protease domain located at the C-terminal fragment.

(2) Human separase can be auto-cleaved into N- and C-terminal fragments, which consist of approximately two-thirds and one-third of the entire protein, respectively. Except for a small region of the N-terminal fragment, which plays regulatory roles, the function of the N-terminus is unknown. Because ARM/HEAT repeats and helical/super-helical structural tetratricopeptide (TPR) repeats mediate protein–protein interaction and the assembly of multi-protein complexes (D’Andrea & Regan, 2003), the N-terminus of separase, which contains 26 ARM/HEAT repeats (Viadiu et al., 2005) or TPR repeats (Winter et al., 2015), may act as a scaffold to help separase play various roles.

(3) In addition to its canonical role in the dissolution of chromosomal cohesin during metaphase to anaphase transition, separase has also been implicated in centrosome cycle, membrane trafficking and DNA-damage repair. To understand the emerging functions of separase better, identification of separase interactors and additional separase substrates are required.

(4) It is well known that cohesin complex regulates gene expression (Zhang & Pati, 2014; Watrin, Kaiser & Wendt, 2016), but it is not clear how cohesin is removed from the regulatory region of the genes once its task is accomplished. Is separase involved in the removal of cohesin during gene expression? If so, how is separase activity regulated? It would be of great interest to investigate whether or not separase participates in gene expression and how it is involved.

(5) Separase is an oncogene, which is overexpressed in multiple human cancers. Overexpression results in aneuploidy and tumorigenesis in animal models. Due to its importance in the cell cycle and DNA damage repair, deregulation of separase would have profound consequences on the fate of cells and on human health. It is not surprising that deregulation of separase could cause human diseases such as cancer and that it could serve as potential therapeutic target.

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