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REVIEW

Csk-homologous kinase (Chk/Matk): a molecular policeman suppressing cancer formation and progression

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Abstract **Aberrant activation of Src-family tyrosine kinases (SFKs) directs initiation of metastasis and development of drug resistance in multiple solid tumors and hematological cancers. Since oncogenic mutations of SFKs are rare events, aberrant activation of SFKs in cancer is likely due to dysregulation of the two major upstream inhibitors: C-terminal Src kinase (Csk) and its homolog Csk-homologous kinase (Chk/Matk). Csk and Chk/Matk inhibit SFKs by selectively phosphorylating the inhibitory tyrosine residue at their C-terminal tail. Additionally, Chk/Matk can also employ a non-catalytic inhibitory mechanism to inhibit multiple active forms of SFKs, suggesting that Chk/Matk is a versatile inhibitor capable of constraining the activity of multiple active forms of SFKs. Mounting evidence suggests that Chk/Matk is a potential tumor suppressor downregulated by epigenetic silencing and/or missense mutations in several cancers such as colorectal and lung carcinoma. In spite of the potential significance of Chk/Matk in cancer, little is known about its structure and regulation. This review focuses on the mechanisms by which Chk/Matk expression and activity is downregulated in cancers. Specifically, we assessed the evidence demonstrating downregulation of Chk/Matk by epigenetic silencing and missense mutations in cancers. The other focus is the tumor suppressive mechanism of Chk/Matk. The final focus of the review is on the clinical applications of the investigations into the mechanism of epigenetic silencing of Chk/Matk expression and the tumor suppressive mechanism of Chk/Matk; specifically we discussed how they can benefit the development of biomarkers for early diagnosis of cancers and specific SFK inhibitors for use as cancer therapeutics.**

Keywords tumour suppressor, protein tyrosine kinase, Src-family kinases, CSK, CHK/Matk, colon cancer

Introduction

Aberrant activation of Src-family kinases (SFKs) contributes to the formation and/or progression of many types of cancers including solid tumors such as colorectal and breast carcinoma and hematological cancers such as chronic myelogenous leukemia. The role of SFKs in colorectal cancer (CRC) has been extensively investigated. It is clear that metastasis and drug resistance of colorectal carcinomas are predominantly driven by the hyper-activation of SFKs (Han et al., 1996; Le and Bast, 2011; Rosenbluh et al., 2012; Sirvent et al., 2012a, 2012b; Touil et al., 2014). Oncogenic mutations of SFKs are rare events. For example, the SFK members Src and Yes are hyper-activated in over 80% of CRC cases (Han et al., 1996; Summy and Gallick, 2003). However, mutations of Src and Yes were found in only ~1% of all CRC cases analyzed (Sirvent et al., 2012a). These findings indicate that over-activation of SFKs is likely caused by dysregulation of their upstream regulators, especially their upstream inhibitor proteins. C-terminal Src kinase (Csk) and its homologous kinase [(Chk), also referred to as megakaryocyte associated tyrosine kinase (Matk)] are major inhibitors of SFKs. They inhibit SFKs by selectively phosphorylating the conserved regulatory tyrosine near the C-terminal tail (referred to as the C-terminal tail tyrosine). In addition to

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phosphorylation of SFKs, Chk/Matk can employ a non-catalytic mechanism to constrain the activity of multiple active forms of SFKs (Chong et al., 2004, 2006). These findings suggest that Chk/Matk is a versatile SFK inhibitor capable of constraining the activity of multiple active forms of SFKs by both the catalytic and non-catalytic mechanism. The functional significance of Chk/Matk is further substantiated by our previous finding that Chk/Matk expression is suppressed in CRC cell lines and human colon cancer biopsies (Zhu et al., 2008).

Unlike Csk, which is widely expressed in most cells, high level of expression of Chk/Matk is restricted to brain cells such as neurons and hematopoietic cells (Bennett et al., 1994). However, moderate to low levels of Chk/Matk was detected in a number of tissues including small intestine, colon, lung and stomach (Human Protein Atlas). Its expression is suppressed in glioma and carcinoma of colon, lung and stomach, suggesting that Chk/Matk is a potential tumor suppressor of these types of cancers (Supplemental Table S1) (Cance et al., 1994; Bhattacharjee et al., 2001; Kim et al., 2004; Sun et al., 2006; Cho et al., 2011). Much is known about the structure and regulation of Csk [reviewed in (Ia et al., 2010; Okada , 2012)]. In contrast, little is known about the structure and regulation of Chk/Matk, which shares ~50% sequence homology with Csk (Klages et al., 1994). This review aims to focus on our current state knowledge of the mechanism of Chk/Matk regulation at both the transcriptional and post-translational level, and the tumor suppressive mechanism of Chk/Matk. Information documented in COSMIC database (Sanger Institute) reveals more than 50 missense mutations of *Chk/Matk* in a wide spectrum of cancers. Predicting how some of these mutations affect the tumor suppressive activity of Chk/Matk is another focus of this review.

Gene structure and isoforms of Chk/Matk

There is large body of evidence suggesting that Chk/Matk expression is suppressed by epigenetic silencing in multiple cancers (refer to the next section for detail). To understand how *Chk/Matk* transcription is regulated epigenetically, we need to examine the structure of *Chk/Matk* gene and search for features of epigenetic regulation. Human *Chk/Matk* gene consists of 11 introns and 13 exons, spanning approximately 8.4 kb of DNA (Fig. 1A). Exon 1 contains the 5′ untranslated sequence and the transcription start site (TSS), which is located 360 bp upstream of the translation initiation site present in exon 2. Exon 4 contains the sequence encoding the SH3 domain, while exons 5 and 6 encode the SH2 domain. Exons 7–13 encode the catalytic domain, with exon 7 harbouring the sequence forming part of the ATP binding site. No TATA box was identified in the *Chk/Matk* sequence; however, a GC-rich box (1–270 bp) lies upstream of the putative promoter region. In addition, various putative transcriptional regulatory sequences, including GATA-1, Sp1, APRE and APRE1 motifs were identified upstream of the TSS and the GC-rich box (Avraham et al., 1995). Whether transcription of the *Chk/Matk* gene is subjected to regulation by the respective transcription factors that bind to these putative regulatory sequences remains unclear.

The GC-rich box near the putative promoter region contains a number of CpG sites. Since Chk/Matk expression is significantly suppressed in a number of tumor types, the CpG sites in the putative GC-rich box could potentially be the *bona fide* CpG islands methylated to cause transcriptional inhibition of the *Chk/Matk* gene. Relevant to this, the *Chk/Matk* promoter has been found to be hypermethylated in CRC, acute lymphocystic leukemia and glioma (Laffaire et al., 2011; Hinoue et al., 2012).

The existence of three mRNA transcripts of *Chk/Matk* gene is reported in the UCSC Genome Browser and Ensembl databases (Fig. 1B). Transcript 1, which encodes a protein of 507 amino acid residues, contains 13 exons and a promoter upstream of the putative TSS. Transcript 2, which encodes a protein of 466 amino acid residues, is generated by alternative splicing at the codon that encodes methionine-42. It lacks the N-terminal region containing exon 1 and the canonical translation initiation site. A third transcript encoding a protein product of 508 residues has been documented in the UCSC Genome Browser. It contains a putative non-canonical promoter that differs from that of Transcripts 1 and 2. However, it is unclear if the protein product encoded by Transcript 3 exists in nature. Both, the canonical promoter (Transcript 1 and 2) and the non-canonical promoter (Transcript 3) contain potential CpG islands of different size (the canonical promoter CpG island size is 1142 bp with a GC content of 62% and an observed-to-expected CpG ratio of 100%; the non-canonical promoter CpG island size is 427 bp, with a GC content of 66% and an observed-to-expected CpG ratio of 82%). Transcript 1 encodes the 56 kDa isoform, $p56^{Chk/Matk}$, which is expressed in multiple non-neuronal cells (Chow et al., 1994); Transcript 2 encodes the 52 kDa isoform, $p52^{Chk/Matk}$, which is expressed exclusively in neurons (Kuo et al., 1994; Kuo et al., 1997). $p56^{Chk/Matk}$contains 41 amino acids at its N terminus that are absent in $p52^{Chk/Matk}$ (Fig. 1C). Previous studies by Nakayama show that the N-terminal region of $p56^{Chk/Matk}$ is important for targeting it to the nucleus and for phosphorylating nuclear substrates to inhibit cell proliferation (Nakayama et al., 2006). Nonetheless, further investigation is needed to ascertain the functional role of the N-terminal 41 residue segment of $p56^{Chk/Matk}$.

Regulation of Chk/Matk expression by epigenetic silencing?

CpG island methylation in the gene promoter is a common

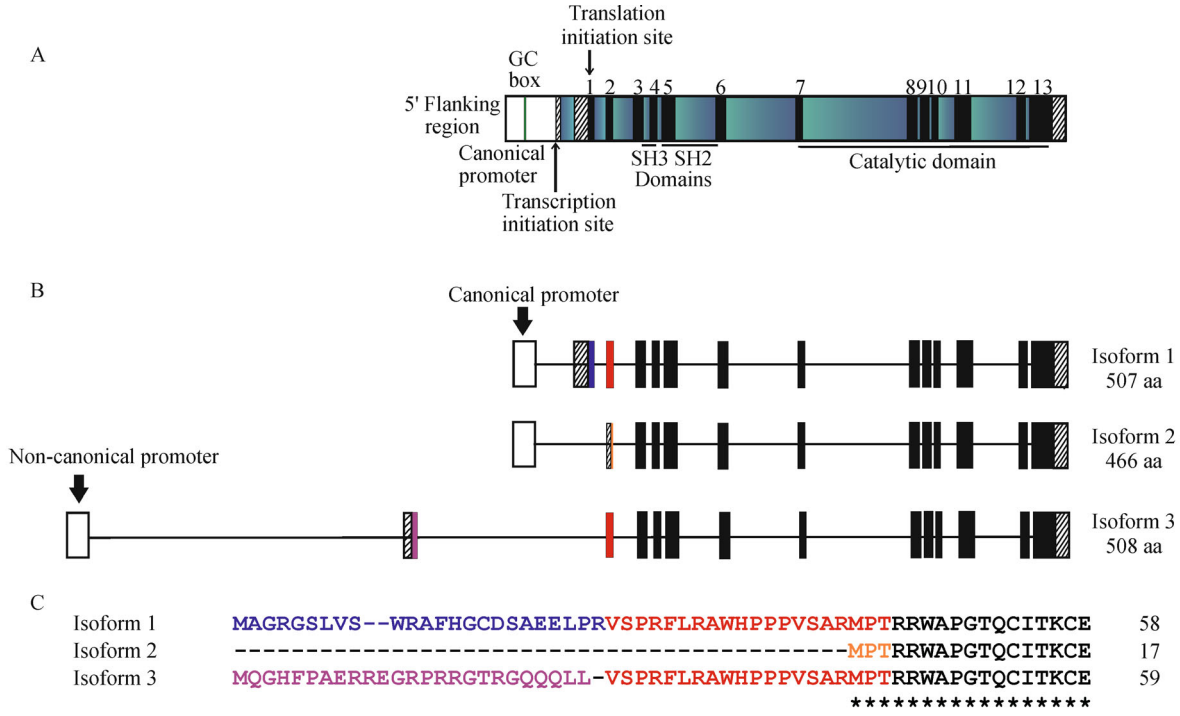

Figure 1 The human *Chk/Matk* gene. (A) Structure of the *Chk/Matk* gene. The *Chk/Matk* gene contains 14 exons of which 13 are protein coding. The transcription initiation site is located 360 bp upstream of the translation initiation site. Shaded boxes are exons which are transcribed but not translated. (B) The three transcripts encoded by the *Chk/Matk* gene. (C) Sequences of the N-terminal segment of the three Chk/Matk isoforms. Among them, isoform two does not contain the first exon and isoform three has a different promoter and a different N-terminal region coded for by exon 1. The sequences in the three putative isoforms (panel C) and the exons from which these sequences are derived (panel B) are presented in the same colors.

mechanism for transcriptional downregulation of tumor suppressor genes in cancers. The mechanism of Chk/Matk regulation has not been sufficiently investigated; however the promoter region of *Chk/Matk* containing multiple transcription factor regulatory elements and potential CpG islands suggests the possible role of epigenetic mechanisms in its regulation.

Using an unbiased genome-wide methylation detection technique [methylated CpG island amplification (MCA) coupled to representational differential analysis (RDA) or DNA promoter microarray], hypermethylation of the *Chk/Matk* promoter was observed in acute lymphocytic leukemia (Kuang et al., 2008). Moreover, *Chk/Matk* was observed to be hypermethylated in low-grade glioma (in 93% of tumor samples) which is consistent with previous reports demonstrating significant downregulation of Chk/Matk in brain tumors (Laffaire et al., 2011). In tissues obtained from colorectal tumors, *Chk/Matk* promoter was found to be significantly hypermethylated in CIMP-positive tumors (Hinoue et al., 2012). Protein expression of Chk/Matk has not been detected in CRC cell lines (Zhu et al., 2008), indicating that its hypermethylation status may govern its regulation. While these preliminary findings have important implications in cancer, further work is needed to unequivocally establish the role of epigenetic silencing in the regulation of Chk/Matk expression.

Regulation of the subcellular localization of Chk/Matk

Chk/Matk lacks the N-terminal fatty acid acylation domain responsible for targeting cellular proteins to the membrane, and thus it is predominantly expressed in the cytosol. Since SFKs are membrane anchored, Chk/Matk needs to be recruited to the plasma membrane to inhibit them. The presence of the SH3 and SH2 domain in Chk/Matk suggests that the binding of protein ligands to these domains can regulate its subcellular localization. Chk/Matk is translocated to specific micro-domains in the plasma membrane upon binding to receptor proteins such as Erb2, c-Kit and TrkA via its SH2 domain. Screening of phosphopeptides libraries revealed that the optimal binding sequence of the SH2 domains of Csk and Chk/Matk are significantly different. The Csk SH2 domain prefers phosphotyrosine containing motifs with the sequence of X-X-pY-(A/S/T)-N-(V/P), where X stands for any amino acid residue, while the optimal binding motif of the Chk/Matk SH2 domain is (I/V/M/L)-(M/E)-pY-

(Y/A/E/T)-M-(A/V/M/I)M (Huang et al., 2008). This supports the notion that the SH2 domains of Chk/Matk and Csk bind to different phosphoproteins and in turn target Chk/Matk and Csk to different subcellular compartments.

Not much is known about the binding specificity of the SH3 domain of Chk/Matk. Generally, the SH3 domain binds to the PxxP motif wherein the two prolines interact with four hydrophobic residues in Csk. However, surprisingly, three of the four conserved residues are substituted with less hydrophobic residues in the Chk/Matk SH3 domain. Whether this unique feature affects the binding specificity of the Chk/Matk SH3 domain remains unclear. The binding of the Chk/Matk SH3 domain to an unidentified protein has been reported to aid in its translocation to the plasma membrane to inhibit the activity of Lyn and suppress cell spreading (Hirao et al., 1998). The SH3 domain of Csk aids in its dimerization, in contrast, Chk/Matk exists as a monomer (Chan et al., 2010), indicating that the Chk/Matk SH3 domain does not cause dimerization. Similar to Csk, Chk/Matk lacks the autophosphorylation site in the activation loop that governs activation of most kinases; therefore tyrosine phosphorylation is probably not responsible for its activation. The mechanism of governing kinase activity of Chk/Matk needs to be further investigated. It is well documented that both the SH3 and SH2 domains of Csk interact with the kinase domain and in turn control the kinase activity of Csk (Huang et al., 2009; Barkho et al., 2013). The N-terminal region of $p56^{Chk/Matk}$ has been reported to aid in nuclear localization of the protein (Nakayama et al., 2006). Interestingly, the nuclear expression of Chk/Matk has been reported to be a novel biomarker for the diagnosis of enteropathy-associated T cell lymphoma (Tan et al., 2011, 2013).

Chk/Matk is a versatile inhibitor capable of suppressing multiple active forms of SFKs

Similar to Csk, Chk/Matk can selectively phosphorylate SFKs at the conserved C-terminal tail tyrosine. Upon phosphorylation, SFKs can adopt the stable inactive conformation, which is stabilized by two intramolecular inhibitory interactions: (i) binding of the phosphorylated C-terminal tail tyrosine to the SH2 domain and (ii) binding of the SH2-kinase linker to the SH3 domain (Tan et al., 1997; Xu et al., 1997). These two interactions constrain the kinase domain in the inactive configuration (Fig. 2A). In normal cells, SFKs are kept at most times in the stable inactive conformation, but can be transiently activated in some cellular events such as mitosis and cell migration [reviewed in (Bjorge et al., 2000)]. SFKs adopting this inactive conformation can be activated by multiple mechanisms including disruption of one of the two intramolecular inhibitory interactions (Moarefi et al., 1997; Lerner and Smithgall, 2002; Lerner et al., 2005), autophosphorylation of the conserved activation loop tyrosine in the kinase domain and dephosphorylation of the conserved C-terminal tail phosphotyrosine [reviewed in (Chong et al., 2005)]. It is noteworthy that for several active forms of SFKs, the kinase domain remains in the active configuration even if the C-terminal tail tyrosine is phosphorylated and remains bound to the SH2 domain. For example, disruption of the interaction between the SH2-kinase linker and SH3 domain by the SH3 domain ligand, and autophosphorylation of the conserved activation loop tyrosine can activate SFKs even if the interaction between the SH2 domain and the phosphorylated C-terminal tail tyrosine remains intact (Fig. 2A) (Moarefi et al., 1997; Lerner and Smithgall, 2002).

In addition to phosphorylating the C-terminal tail tyrosine of SFKs, Chk/Matk is capable of employing another mechanism to suppress SFK activity. This mechanism, independent of its ability to phosphorylate the SFK C-terminal tail tyrosine, involves direct binding of the Chk/Matk kinase domain to the kinase domain of multiple active forms of SFKs (Chong et al., 2004, 2006). The binding alone is sufficient to suppress the SFK activity. Thus, this mechanism is referred to as the non-catalytic inhibitory mechanism of Chk/Matk. Despite the high degree of sequence homology between Csk and Chk/Matk, Csk cannot adopt this inhibitory mechanism to inhibit SFKs *in vitro* (Chong et al., 2006). These findings suggest that Chk/Matk is a versatile upstream SFK inhibitor capable of suppressing activity of multiple active forms of SFKs by phosphorylating SFKs and also by the non-catalytic inhibitory mechanism. Furthermore, these findings imply that Csk alone is not able to constrain activity of all active SFKs in cells. Indeed, Zhu, *et al.* found that Chk/Matk expression is suppressed in all the CRC cell lines they examined; the lack of Chk/Matk expression contributes to constitutive activation of Src even though Csk is expressed at a relatively high level (Zhu et al., 2008). In contrast to the inability of Csk to compensate for the lack of Chk/Matk in colon cancer cells, Chk/Matk is able to compensate for the lack of Csk in mouse embryo fibroblasts deficient of Csk-Chk/Matk was able to reduce the number of phosphotyrosine containing proteins as well as reducing the increasing activity of Src and Fyn (Davidson et al., 1997). The determinants in Chk/Matk governing its non-catalytic inhibitory activity have not clearly been defined. Previously, we have mapped the determinants governing high affinity binding of SFKs to the kinase domain of Chk/Matk (Chong et al., 2006). Intriguingly, a point mutation in the ATP binding site of Chk/Matk abolishes the kinase activity as well as its ability to bind and inhibit SFKs with the non-catalytic inhibitory mechanism (Chong et al., 2004). These results suggest that Chk/Matk needs to bind ATP to achieve a conformation compatible with the inhibition of SFKs with the non-catalytic mechanism.

Future investigation to define the structural basis of this non-catalytic inhibitory mechanism of Chk/Matk will benefit the development of a new generation of specific inhibitors to suppress the oncogenic activity of SFKs.

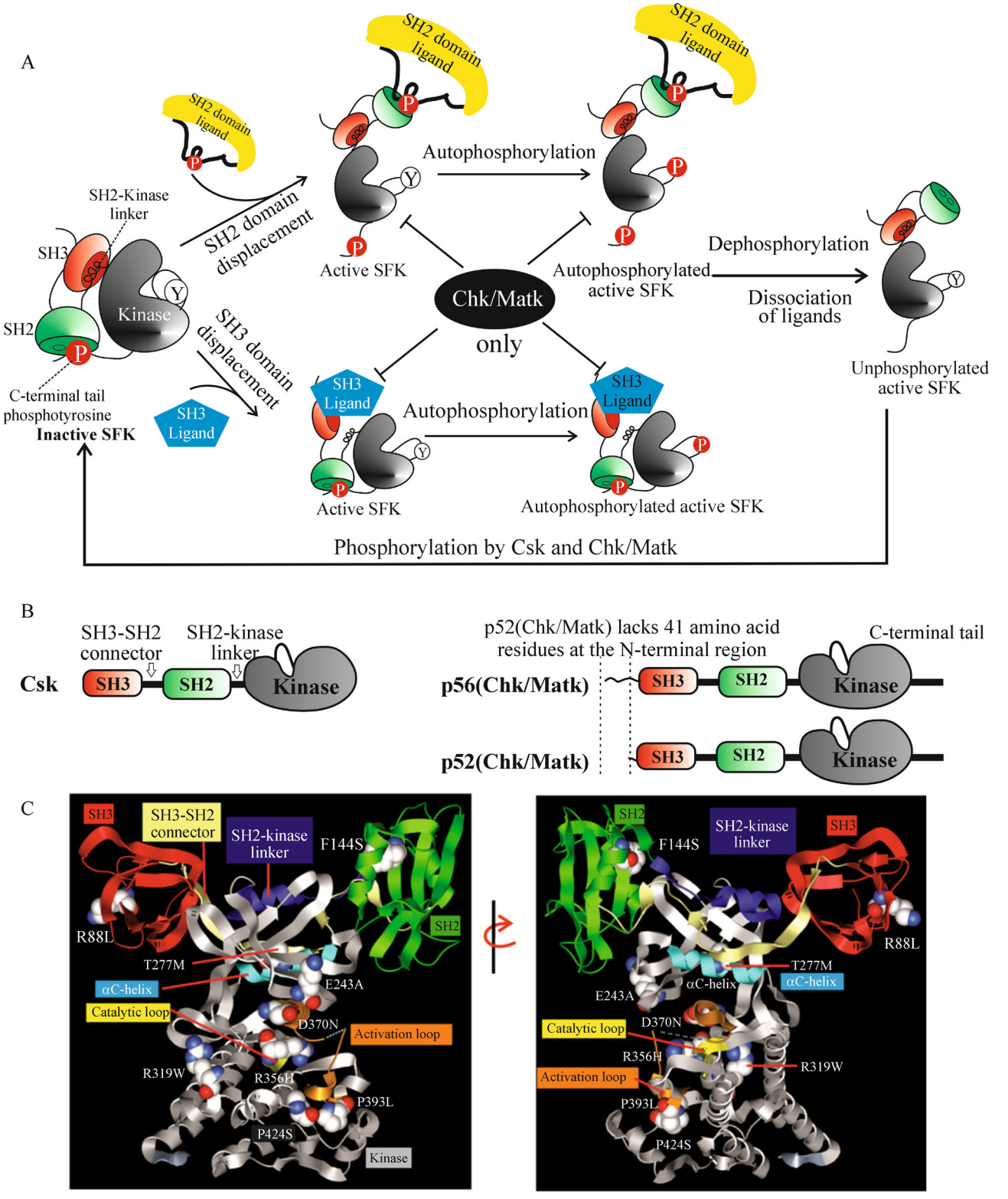

Figure 2 The non-catalytic inhibitory mechanism of Chk/Matk and a model of Chk/Matk structure showing the locations of cancer-associated missense mutations. (A) A schematic diagram depicting how SFKs are activated to form multiple active forms by binding to the ligands of the SH2 and SH3 domains and by autophosphorylation. Biochemical analysis demonstrated that Chk/Matk but not Csk can employ the non-catalytic inhibitory mechanism to bind and inhibit all the active forms of SFKs. Dephosphorylation by specific phosphatases and dissociation of the ligands covert these active forms of SFKs to the unphosphorylated/unliganded form. Csk and Chk/Matk can induce this form of SFKs to adopt the stable inactive conformation by phosphorylation of the C-terminal tail tyrosine. (B) A schematic diagram showing the similarities and differences of CSK and the two isoforms of Chk/Matk $p52^{Chk/Matk}$ and $p56^{Chk/Matk}$. (C). Using the Csk structure as the model, the locations of key residues found to undergo missense mutation in cancers are shown.

Chk/Matk is a potential tumor suppressor of multiple types of cancer

Over-activation of SFKs in intestinal stem cells can direct CRC tumorigenesis in both *Drosophila* and mouse models (Cordero et al., 2014). Although it is unclear how SFKs are over-activated in intestinal stem cells, expression of recombinant human Chk/Matk suppresses hyper-proliferation of *Drosophila* intestinal stem cells caused by over-activation of the SFK members, suggesting that Chk/Matk is a potential upstream regulator of SFKs in intestinal stem cells. Relevant to this, Chk/Matk is normally expressed in colon epithelial cell lines and colon tissues (Zhu et al., 2008), but its expression is significantly reduced in CRC cell lines and colon cancer biopsies. Importantly, overexpression of Chk/Matk in the DLD-1 CRC cell line inhibits Src activity, suppresses anchorage-independent growth and cell migration (Zhu et al., 2008). All these findings suggest that Chk/Matk is a potential CRC tumor suppressor. Future investigation can focus on determining whether Chk/Matk suppresses CRC tumorigenesis by preventing over-activation of SFKs in mammalian intestinal stem cells. How Chk/Matk expression is downregulated remains unclear. *Chk/Matk* promoter is hypermethylated in CRC (Hinoue et al., 2012). Presumably, the hypermethylation suppresses Chk/Matk expression by inhibiting transcription of *Chk/Matk* gene.

In addition to possible downregulation by epigenetic silencing, Chk/Matk can be dysregulated by missense mutations in various types of cancer. So far, over 50 cancer-associated missense mutations have been listed in the COSMIC database of Sanger Institute. Much is known about the structural basis of regulation of Csk and how Csk recognizes Src as a substrate (Takeuchi et al., 2000; Ogawa et al., 2002; Matsuoka et al., 2004; Wong et al., 2005; Lee et al., 2006; Lieser et al., 2006; Levinson et al., 2008; Huang et al., 2009; Ia et al., 2010; Jamros et al., 2012; Okada, 2012; Barkho et al., 2013). Given the high degree of sequence homology between Csk and Chk/Matk, it is possible to use the Csk structure as a model to predict the impact of each of the missense mutations on the kinase activity, substrate recognition, regulation and tumor suppressive activity of Chk/Matk. Supplemental Figure S1 shows the alignment of the sequences of Csk and Chk/Matk with the cancer-associated mutated residues of Chk/Matk highlighted. A number of the mutated residues are mapped to the motifs that are potentially critical to catalysis and recognition of SFKs. The locations of homologous residues of these mutated amino acids in the Csk structure are shown in Fig. 2C. For example, E243A is mapped to the glycine-rich loop which is critical for binding of the β- and γ-phosphate of ATP; D370 is mapped to the DFG motif critical for catalysis and binding to the magnesium ion complexed with ATP. These mutations likely perturb the ability of Chk/Matk to bind ATP and catalyze phosphorylation of SFKs. R319W and P424S are mapped to the putative Src binding motifs. These mutations likely impact the ability of Chk/Matk to phosphorylate SFKs and/or inhibit SFKs by the non-catalytic inhibitory mechanism. A summary of the predicted impacts of the cancer-associated missense mutations on the kinase activity and tumor suppressive action of Chk/Matk is presented in Supplemental Table S2. It is obvious from our prediction that most of these missense mutations can abolish or reduce the kinase activity and SFK binding ability. Our prediction further supports the notion that downregulation of Chk/Matk expression and/or function contributes to over-activation of SFKs and in turn contributes to tumorigenesis and progression of a number of cancers.

Summary

Mounting evidence suggests Chk/Matk as a potential tumor suppressor downregulated in multiple types of cancer by epigenetic silencing and missense mutations. Future investigation should focus on deciphering the molecular basis of epigenetic silencing of Chk/Matk expression. One approach to achieve this aim is to define the CpG sites in the *Chk/Matk* promoter that are constitutively methylated to suppress transcription of the *Chk/Matk* gene in cancer cells. Once these CpG sites are defined, methylation-specific PCR to monitor methylation levels of the CpG sites can be exploited for the development of biomarkers for early diagnosis of CRC. Relevant to this, methylation specific PCR of the *vimentin* gene promter has been developed as a diagnostic test for population-based screening program for early detection of CRC.

In addition to DNA methylation, histone modifications by histone deacetylases (HDACs) also contribute to epigenetic silencing. The role of HDACs in the regulation of *Chk/Matk* gene transcription can be investigated using HDAC knockout mice and monitoring the impact of specific HDACs on Chk/Matk expression. Future investigation is needed to clearly define how Chk/Matk activity is regulated and how Chk/Matk exerts its tumor suppressive activity. One approach to achieve this is to express recombinant Chk/Matk in Chk/Matk-deficient cancer cells such as CRC cell lines, and then monitor changes in the global proteome and phosphoproteome of the cancer cells caused by expression of recombinant Chk/Matk. Finally, how the non-catalytic inhibitory mechanism of Chk/Matk inhibits SFKs remains unclear. This question can be addressed by solving the crystal structure of the Chk/Matk complexed with a SFK member such as Src. More importantly, defining the structural basis of the non-catalytic inhibitory mechanism of Chk/Matk will benefit the development of a new generation of selective SFK inhibitors as anti-tumor therapeutics to induce growth arrest and/or halt migration of cancer cells. In addition to phosphorylating and inhibiting SFKs, Chk/Matk can selectively phosphorylate and modulate the activity of other cellular proteins. For example, β-synuclein in brain tissue lysate was found to be a

preferential substrate of Chk/Matk (Ia et al., 2011). Future investigation may include deciphering the role of these non-SFK substrates in the tumor suppressive action of Chk/Matk.

Compliance with ethics guidelines

Gahana Advani, Anderly Chueh, Ya Chee Lim, Amardeep Dhillon and Heung-Chin Cheng declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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