The VASP-Spred-Sprouty Domain Puzzle

Published, JBC Papers in Press, September 20, 2006, DOI 10.1074/jbc.R600021200
Karina Bundschu1,2, Ulrich Walter3, and Kai Schuh4

From the 1Abteilung Biochemie und Molekularbiologie, Universität Ulm, 89081 Ulm, 2Institut für Klinische Biochemie und Pathobiologie, Universität Würzburg, 97080 Würzburg, and 3Physiologisches Institut, Universität Würzburg, 97070 Würzburg, Germany

Sprouty-related proteins with an EVH1 domain (Spreds) belong to a new protein family harboring a conserved N-terminal EVH1 domain, which is related to the VASP (vasodilator-stimulated phosphoprotein) EVH1 domain (Enabled/VASP homology 1 domain) and a C-terminal Sprouty-related domain, typical for Sprouty proteins. Spreds were, like Sprouts, initially discovered as inhibitors of the Ras/MAPK pathway, and the SPR (Sprouty-related) domains of both protein families seem to be very important for many protein interactions and cellular processes. VASP was initially characterized as a proline-rich substrate of protein kinases A and G in human platelets and later characterized as a proline-rich substrate of protein kinases A and G in human platelets and later shown to be a scaffold protein, regulating both signal transduction pathways and the actin filament system. The VASP-EVH1 domain is known to bind specifically to a FP4 binding motif, which is, for example, present in the focal adhesion proteins vinculin and zyxin. In this review we give a structural and functional overview on these three protein families and ask whether nature plays a modular protein domain puzzle with stable exchangeable elements or if these closely related domains have various functions when pasted in a different protein context.

Family Members, Structure, and Expression

Sprouty—Sprouty proteins contain, like Spreds, a highly conserved cysteine-rich domain at the C terminus (21–25) and are inhibitors of the MAPK signaling pathway. The N-terminal regions of Sprouty proteins are astonishingly variable, but they contain a conserved SH2 domain binding motif (NXXXXXP) including a central tyrosine residue (Tyr-55 in Sprouty2) (26, 27). So far, Drosophila Sprouty, Xenopus Sprouty, avian Sprouty (chick Sprouty2), and four mammalian isoforms have been identified (21–25, 28). Interaction partners of Sprouts are c-Cbl (cellular homologue of Casitas B lineage lymphoma proto-oncogene product), Grb2 (growth factor receptor-bound 2), Gap1 (GTPase-activating protein), FRS2 (fibroblast growth factor receptor substrate 2), SHP-2 (Src homology 2 domain-containing protein-tyrosine phosphatase 2), Raf1, TESK1 (testis-specific protein kinase-1), caveolin-1, and PTP1B (protein-tyrosine phosphatase 1B) (for review see Refs. 29 and 30), Mammalian VASP was first characterized in platelets as a proline-rich substrate of cAMP- and cGMP-dependent protein kinases (11). Additional members of the Ena/VASP family are the Drosophila Enabled (Ena), its mammalian homologue Mena (mammalian Enabled), the C. elegans homologue unc34, and the murine Evl (Enabled/vasodilator-stimulated phosphoprotein-like) (12–14). In general, the EVH1 domain is followed by a central proline-rich region responsible for interaction with Src homology 3 domains and profilins (compiled in Ref. 15) and, finally, by an EVH2 domain, mediating binding to G- and F-actin (15–18). Because of their important role in actin cytoskeleton organization, members of the Ena/VASP family are expressed most likely in almost all mammalian cell types (11, 19). VASP is associated to actin filaments and localized in focal adhesion complexes, dynamic membrane regions, leading membrane areas of non-epithelial cells, and cell/cell contacts of Madin-Darby canine kidney cells (12). Drosophila Enabled, Mena, and unc34 are localized in axonal structures and are important during neuronal development (13, 20).

Sprouty—Sprouty proteins contain, like Spreds, a highly conserved cysteine-rich domain at the C terminus (21–25) and are inhibitors of the MAPK signaling pathway. The N-terminal regions of Sprouty proteins are astonishingly variable, but they contain a conserved SH2 domain binding motif (NXXXXXP) including a central tyrosine residue (Tyr-55 in Sprouty2) (26, 27). So far, Drosophila Sprouty, Xenopus Sprouty, avian Sprouty (chick Sprouty2), and four mammalian isoforms have been identified (21–25, 28). Interaction partners of Sprouts are c-Cbl (cellular homologue of Casitas B lineage lymphoma proto-oncogene product), Grb2 (growth factor receptor-bound 2), Gap1 (GTPase-activating protein), FRS2 (fibroblast growth factor receptor substrate 2), SHP-2 (Src homology 2 domain-containing protein-tyrosine phosphatase 2), Raf1, TESK1 (testis-specific protein kinase-1), caveolin-1, and PTP1B (protein-tyrosine phosphatase 1B) (for review see Refs. 29 and 30), whereas the latter four bind to the C-terminal part of the protein. Binding to Caveolin-1 targets Sprouty, like Sprouty, to membrane microdomains (31), TESK1 binding suppresses cell spreading by inhibiting the kinase activity of TESK1 (32), and PTP1B binding inhibits cell migration (33). Murine Sprouty1, -2, and -4 are widely expressed in embryonic and adult tissues (22–24), whereas Sprouty3 expression is restricted to adult brain and testis (24, 34). In stimulated cells, Sprouty proteins are distributed unevenly in the cytoplasm, except of human Sprouty2, which is additionally colocalized with microtubules. Following ligand-dependent RTK activation, a subpopulation translocates to the leading edge of the plasma membrane (31, 35–37).
MINIREVIEW: VASP-Spred-Sprouty Review

Domains: Structure and Functions

**EVH1**—So far, four EVH1 families are known, the Ena/VASP, the Wiskott-Aldrich-Syndrome (WASP), the Homer/Vesl, and the Spred family. The EVH1 domain specifically binds proteins with proline-rich sequences (38–41), thereby playing an important role in the integrity of the cytoskeleton, actin-based cell motility, postsynaptical signal transduction, cell proliferation, and differentiation (reviewed in Refs. 15 and 42).

The EVH1 domain of the Ena/VASP family recognizes the distinct (E/D)FPPPP bind motif (40, 43). This motif is regarded as a protein binding site, present in the focal adhesion proteins vinculin (39, 40) and zyxin (40), the axon guidance protein roundabout (Robo) (44), lipoma preferred partner (LPP) (45), semaphorin 6A-1 (Sema6A-1) (43), T-cell signaling Fyn-binding protein/SLP-76-associated protein (Fyb/SLAP) (46), and the surface protein ActA of Listeria (40).

In Spred proteins, the N-terminal EVH1 domain seems to play an essential role in the inhibition of ERK activation (4, 5, 47). Recently, structural information on the Spred EVH1 domain revealed that this domain adopts, like the other EVH1 domains, a pleckstrin homology fold (48, 49). However, for the Spred EVH1 domain a different induced fit peptide binding mechanism has been proposed even in the absence of a characterized ligand for this domain (48, 49), which makes the identification of binding partners a point of major interest (Fig. 1).

**KBD**—KBD in the middle part of the Spred proteins consists of about 50 amino acids (amino acids 234–286) and is not related to any previously identified tyrosine kinase interaction domains (5). Spred-3 with a non-functional KBD and deletion mutants of Spred-1 in this region revealed that this internal region is involved but not essential for ERK suppression (2, 5, 50). This raises the question of what the physiological function of this domain might be.

**SPR**—Spred and Sprouty proteins harbor a well conserved cysteine-rich SPR domain at the C terminus. In the case of Spred-2, it is required for efficient suppression of stem cell factor-induced ERK phosphorylation and suppression of hematopoietic cell development (51). A C-terminal deletion mutant of Spred-1 was shown to act as a dominant negative form and augments growth factor-induced ERK activation (5, 50). Contrary data were recently published by King et al. (47), where ΔC-Spred-2 was unable to suppress ERK activation but ΔC-Spred-1 and Eve-3 were still functional in suppressing ERK activation (4, 47). Therefore, future work has to be done, elucidating the physiological function of the SPR domain.

The SPR domain of Spred and Sprouty proteins is thought to be palmitoylated and necessary for membrane localization after growth factor stimulation (2, 5, 31, 37, 52, 53). Additionally, the minimal membrane-targeting region of the SPR domain in both protein families is necessary and sufficient for plasma membrane localization and binding to phosphatidylinositol 4,5-bisphosphate (37). Moreover, mutations disrupting this region interfere with their functions in modulating cell migration, cell proliferation, and ERK/MAPK activation (52, 53). All four mammalian Sprouty proteins are able to form hetero- and homo-oligomers via their C-terminal SPR domains (54), and although each of the Sprouty isoforms by itself inhibits growth factor-induced activation of the ERK pathway, hetero-oligomers show a more pronounced inhibitory activity (54). Recently, Spred-1 and Spred-2 were also presented to form heterodimers via their SPR domains, which increases the variety of regulatory possibilities (47).

**Signaling Pathways and Physiological Functions**

**Spred**—Spreds were first described as suppressors of the Ras/Raf/MAPK signaling pathway (5). They were constitutively associated with Ras, but the inhibitory effect was exerted by suppressing the phosphorylation and activation of Raf (5). King et al. (47) located the target upstream of Ras, suggesting different interaction modes dependent on the cellular context. Spreds are potent inhibitors of a wide range of mitogenic stimuli like different growth factors, cytokines, and chemokines, but the suppressing effect seems to be restricted to the Ras/ERK/MAPK pathway (2, 7, 47, 50, 55).

In vivo studies using knock-out mouse models begin now to uncover the physiological Spred functions. Spred-1/−/− and Spred-2/−/− mice revealed that these proteins are not existentially necessary for fertility and development, and young adult mice are viable and show no apparent abnormalities (56, 57). However, loss of functional Spred-2 protein causes a dwarf phenotype, similar to hypochondroplasia, a common form of human dwarfism (56). In this context, Spred-2 seems to be an important modulator of bone morphogenesis by inhibiting the fibroblast growth factor-induced MAPK pathway (56). A similar phenotype was also mentioned for Spred-1/−/− mice (57), underlining the role of Spred proteins in growth regulation and supporting the idea of overlapping physiological functions. Furthermore, Spreds seem to be important for hematopoiesis. In Spred-2/−/− midgestation mouse embryos, Spred-2 suppresses hematopoietic processes by inhibiting MAPK activation (51),...
and stem cell factor or interleukin 3 stimulation of mature Spred-1−/− bone marrow-derived mast cells resulted in increased cell proliferation and MAPK activation (50). Therefore, Spred-2 could serve as a negative regulator of embryonic (51) and Spred-1 of mature late phase hematoepoiesis, respectively (50). Moreover, Spred-1 seems to play a role in the regulation of eosinophils during allergic asthma processes (57). A specific function of Spred-3, which is expressed exclusively in brain (2), is not known yet.

VASP—Members of the Ena/VASP family are regarded as regulators of the actin cytoskeleton. Ena/VASP proteins promote actin polymerization and assembly, and several mechanisms have been proposed, which include the regulation of nucleation, bundling, branching, and, perhaps somewhat controversial, capping. However, the precise mechanisms remain to be elucidated and may also vary with the cell type and model used (15, 58, 59). At the molecular level, VASP proteins are considered as some kind of adapters, linking the proteins binding to the EVH1 domain to the actin polymerization process, which is regulated by the EVH2 domain or vice versa, guiding the actin polymerization machinery to intracellular spaces predetermined by EVH1-interacting proteins (13, 17, 40, 45, 60, 61).

Phosphorylation by protein kinase A and G at the site within the VASP EVH2 domain (Ser-239) but not at Ser-153/157 is established to inhibit F-actin binding/bundling by VASP (62, 63), which may also be caused by AMP-dependent kinase-mediated phosphorylation of VASP threonine 278.3 Moreover, the functions of VASP-like proteins can be adjusted to the particular cellular needs by protein kinase C phosphorylation (Ser-157) (64) or by several phosphatases (65). A further level of actin cytoskeleton regulation was added by the observation that SH3 domains interact with the central proline-rich region (for review see Ref. 15), located between the two EVH domains, thereby influencing a variety of pathways. The profilin/G-actin complexes are also recruited by this central region (66), thereby bringing these complexes in close proximity to the nascent filamentous actin bundle emerging from the flowering bouquet-like tetramer of VASP-like proteins, held together by the tetramerization domains at the C termini of EVH2 domains (16, 67).

In vertebrates, genetic analysis of Ena/VASP function was hampered by the broad and overlapping expression of the three highly related family members Mena, VASP, and EVL (Ena/VASP-like). Mice deficient in either Mena or VASP exhibit subtle defects in forebrain commissure formation and platelet activation, respectively (68–71). Mena−/−/VASP−/− double mutants die perinatally and display defects in neurulation, in development of craniofacial structures, and in the formation of several fiber tracts in the central and peripheral nervous system (72). It is likely that the expression of the third family member, EVL, masks the requirement for Ena/VASP function in other cell types of Mena−/−/VASP−/− animals.

Sprouty—Like Spreds, members of the Sprouty family negatively regulate ERK activation (21, 73, 74). However, the related Sprouty proteins seem to differ in their pathway specificity and regulate other signaling pathways in addition to ERK (28, 31).

Sprouty can target RTK signaling at many levels, upstream of ERK/MAPK and downstream of RTKs, and the exact position of Sprouty in the pathway is probably dependent on the cellular context (for review see Ref. 30). Compared with Spreds, mammalian Sproutys are more selective inhibitors, blocking fibroblast growth factor- but not epidermal growth factor-induced ERK activation (31, 55, 75, 76). Moreover, Sprouty family members regulate the RTK signaling not only negatively but also positively (reviewed in Refs. 77 and 78). This effect might be due to the large differences in the N-terminal areas of these proteins, but the molecular mechanisms need further clarification.

Sproutys act as antagonists in many physiological and developmental processes and play a role in cell proliferation, cell motility, and receptor trafficking. In mammals, Sproutys regulate different developmental branching processes, like lung development (79), angiogenesis (35, 80), and placenta morphogenesis (81). Moreover, they play a role during human follicle maturation (82) and myogenic differentiation (83). Surprisingly, during chick bone development (24), a contrary phenomenon was observed compared with Spred-2-null mice (56).

Here, the overexpression of Sprouty (24) and not the loss of Sprouty (56) led to a hypochondroplasia-like phenotype. Therefore, Sprouty1 and Sprouty may have different regulatory functions during this developmental process.

Phosphorylation of Sproutys is not only observed but also necessary for physiological functions (27, 52, 55, 84–86). The phosphorylation of the N-terminal Tyr-55 in Sprouty2 is necessary for the interaction with and acts as binding site for the SH2 domains of c-Cbl (27) and Grb2 (52, 76, 86). Besides this, the phosphorylation of C-terminal tyrosine residues seems to be important for the specific Sprouty inhibition (87). An additional regulatory mechanism of Sproutys is the recently discovered mitogen-activated protein kinase-interacting kinase 1 (Mnk1)-mediated serine phosphorylation, in which serine phosphorylation stabilizes Sprouty2 (88).

Furthermore, Sprouty activity is controlled by SHP-2 tyrosine phosphatases, which dephosphorylate the critical tyrosine of Sproutys (89).

Knock-out mouse models begin now to bring some light into Sprouty in vivo functions. Sprouty1−/− mice displayed multiple ureteric and kidney failures due to increased sensitivity of the Wolffian duct to glial cell line-derived neurotrophic factor (GDNF)/RET signaling (90). Astonishingly, Sprouty2−/− mice did not show renal defects but suffer from enteric nerve hyperplasia and hypergangliosis resulting in esophageal achalasia, dilated esophagus, intestinal pseudo-obstruction, and abnormal physiology of the digestive motility system (91). Sprouty1−/− and Sprouty2−/− mice displayed both premature death and had, like the Spred-2−/− mice, a reduced body size (91). Another study confirmed these abnormalities, whereas these mice also had severe hearing loss with an abnormal Corti organ and abnormal cochlear hair cell morphology (92).

Summary and Future Perspectives

Here, we gave an overview of the three closely related protein families VASP, Spred, and Sprouty. Compared with VASP, Spred and Sprouty share a more similar overall domain organization, and comparison of both SPR domains revealed some overlapping functions. Thus, nature may use a restricted mod-

3 C. Blume, personal communication.
ular domain system in similar protein families as building blocks for the fine tuning of a variety of developmental processes, but there are still a lot of important unanswered questions on which future work should be focused.

Ligands and the Role of the Spred EVH1 Domain?—So far, no ligands were discovered for the Spred-EVH1 domain, but the three-dimensional structure of the Spred-EVH1 domain suggests a binding mode differing from all known EVH1 domains.

Role of the Spred KBD?—As Spred-3 with a non-functional KBD and deletion mutants of Spred-1 in this region are still functional in ERK suppression, the specific modular role of this domain is still unclear. An interesting point to follow might be the potential involvement in c-Kit-mediated signaling (93), which was shown to be altered in malignant growth, and c-Kit is also regarded as a marker for interstitial pace-making Cajal cells in the digestive system and in the urogenital tract (94, 95).

Functions of Spred and Sprouty in the Ras/MAPK Pathway?—Spreds and Sproutys are inhibitors of the Ras/MAPK pathway, and both groups seem to be able to inhibit at different levels, surely dependent on the intracellular context. Detailed clarification of the spatial distribution, and functional interactions will be very important for a deeper understanding. Sproutys can also positively regulate RTK signaling, but such mechanisms will be very important for a deeper understanding. Sproutys can surely dependent on the intracellular context. Detailed clarification could be an additional regulatory mechanism in Spreds.

Regulatory Mechanisms?—For VASP and Sprouty proteins, the phosphorylation state regulated by different kinases and phosphatases at different phosphorylation sites was shown to be functionally very important. However, whether the known sites and kinases are already complete and if phosphorylation plays an important role for Spred regulation as well are important future tasks to clarify. Furthermore, hetero-/homo-oligomerization could be an additional regulatory mechanism in Spreds.

In Vivo Roles of VASP, Spred, and Sprouty?—Recently, knock-out mouse models of all three protein families started to shed some light into their physiological functions. However, overlapping expression of highly related family members hamper the studies. Therefore, the combination of conventional and conditional knock-out mouse models to circumvent embryonic lethality of double or even triple knock-outs will enlighten the physiological functions of these protein families.

Considering all data, there is no general structural or functional model fitting all three protein families. A principle role of the VASP-EVH1 domain is to recruit an actin filament regulator to cellular sites where a spatial and temporal regulation of actin polymerization is required. Therefore, we postulate that the Spred-EVH1 domain could be required to recruit an important inhibitor of the Ras/ERK/MAPK pathway to cellular sites where this inhibition will be essential. The identification of Spred-EVH1 ligands will begin to unravel this important question.

REFERENCES
1. DeMille, M. M., Kimmel, B. E., and Rubin, G. M. (1996) Gene (Anst.) 183, 103–108
2. Kato, R., Nonami, A., Taketomi, T., Wakioka, T., Kuroiwa, A., Matsuda, Y., and Yoshimura, A. (2003) Biochim. Biophys. Res. Commun. 302, 767–772
3. Sivak, J. M., Petersen, L. F., and Amaya, E. (2005) Dev. Cell 8, 689–701
4. King, I. A., Corcoran, N. M., D’Abaco, G. M., Straffon, A. F., Smith, C. T., Poon, C. L., Buchert, M., I. S., Hall, N. E., Lock, P., and Hovens, C. M. (2006) J. Hepatol. 44, 758–767
5. Wakioka, T., Sasaki, A., Kato, R., Shouda, T., Matsumoto, A., Miyoshi, K., Tsuneoka, M., Komiya, S., Baron, R., and Yoshimura, A. (2001) Nature 412, 647–651
6. Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M., and Yoshimura, A. (2003) Nat. Cell Biol. 5, 427–432
7. Miyoshi, K., Wakioka, T., Nishinakamura, H., Kamio, M., Yang, L., Inoue, M., Hasegawa, M., Yonemitsu, Y., Komiya, S., and Yoshimura, A. (2004) Oncogene 23, 5567–5576
8. Nonami, A., Taketomi, T., Kimura, A., Saeki, K., Takahai, H., Sanada, T., Taniguchi, K., Harada, M., Kato, R., and Yoshimura, A. (2005) Genes Cells 10, 887–895
9. Engelhardt, C. M., Bursch, K., Messerschmitt, M., Renne, T., Walter, U., Reinhard, M., and Schuh, K. (2004) Histochem. Cell Biol. 122, 527–538
10. Bursch, K., Gattenlohe, S., Knobeloch, K. P., Walter, U., and Schuh, K. (2006) Gene Expr. Patterns 6, 247–255
11. Halbrugge, M., and Walter, V. (1989) Eur. J. Biochem. 185, 41–50
12. Haffner, C., Jarchau, T., Reinhard, M., Hoppe, J., Lohmann, S. M., and Walter, U. (1995) EMBO J. 14, 19–27
13. Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P. (1996) Cell 87, 227–239
14. Lanier, L. M., and Gertler, F. B. (2000) Curr. Opin. Neurobiol. 10, 80–87
15. Reinhard, M., Jarchau, T., and Walter, U. (2001) Trends Biochem. Sci. 26, 243–249
16. Bachmann, C., Fischer, L., Walter, U., and Reinhard, M. (1999) J. Biol. Chem. 274, 23549–23557
17. Huttelmaier, S., Harbeck, B., Steffens, O., Messerschmidt, T., Illenberger, S., and Jockusch, B. M. (1999) FEBS Lett. 451, 68–74
18. Harbeck, B., Huttelmaier, S., Schluter, K., Jockusch, B. M., and Illenberger, S. (2000) J. Biol. Chem. 275, 30817–30825
19. Reinhard, M., Zumbrunn, J., Jaquemar, D., Kuhn, M., Walter, U., and Trubel, B. (1999) J. Biol. Chem. 274, 13410–13418
20. Colavita, A., and Culotti, J. G. (1998) Dev. Biol. 194, 72–85
21. Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., and Krasnow, M. A. (1998) Cell 92, 253–263
22. Tefft, J. D., Lee, M., Smith, S., Leinwand, M., Zhao, J., Bringas, P., Jr., Crowe, D. L., and Warburton, D. (1999) Curr. Biol. 9, 219–222
23. de Maximy, A. A., Nakatake, Y., Moncada, S., Itoh, N., Thiery, J. P., and Belluscio, S. (1999) Mech. Dev. 81, 213–216
24. Minowada, G., Jarvis, L. A., Chi, C. L., Neubuser, A., Sun, X., Hacohen, N., Krasnow, M. A., and Martin, G. R. (1999) Development 126, 4465–4475
25. Chambers, D., and Mason, I. (2000) Mech. Dev. 91, 361–364
26. Guy, G. R., Wong, E. S., Yusoff, P., Chandramouli, S., Lo, T. L., Lim, J., and Fong, C. W. (2003) J. Cell Sci. 116, 3061–3068
27. Rubin, C., Litvak, V., Medvedovsky, H., Zwang, Y., Lev, S., and Yarden, Y. (2003) Curr. Biol. 13, 297–307
28. Nutt, S. L., Dingwell, K. S., Holt, C. E., and Amaya, E. (2001) Genes Dev. 15, 1152–1166
29. Dikic, I., and Giordano, S. (2003) Curr. Opin. Cell Biol. 15, 128–135
30. Kim, H. J., and Bar-Sagi, D. (2004) Nat. Rev. Mol. Cell. Biol. 5, 441–450
31. Impagnatiello, M. A., Weitzer, S., Gannon, G., Compagni, A., Cotten, M., and Christofori, G. (2001) J. Cell Biol. 152, 1087–1098
32. Tsumura, Y., Toshima, J., Leeksma, O. C., Ohashi, K., and Mizuno, K. (2005) Biochem. J. 387, 627–637
33. Yigzaw, Y., Poppleton, H. M., Sreejayan, N., Hassid, A., and Patel, T. B. (2003) J. Biol. Chem. 278, 284–288
34. Leeksma, O. C., Van Achterberg, T. A., Tsumura, Y., Toshima, J., Eldering, E., Kroes, W. G., Mellink, C., Spaargaren, M., Mizuno, K., Pannekoek, H., and de Vries, C. J. (2002) Eur. J. Biochem. 269, 2546–2556
35. Lee, S. H., Schloss, D. J., Jarvis, L., Krasnow, M. A., and Swain, J. L. (2001) J. Biol. Chem. 276, 4128–4133
36. Lim, J., Wong, E. S., Ong, S. H., Yusoff, P., Low, B. C., and Guy, G. R. (2000) J. Biol. Chem. 275, 32837–32845
37. Lim, J., Yusoff, P., Wong, E. S., Chandramouli, S., Lao, D. H., Fong, C. W.,
