The regulation and function of the NUAK family

Xianglan Sun¹, Ling Gao², Hung-Yu Chien³, Wan-Chun Li^{4,5} and Jiajun Zhao¹

¹Department of Endocrinology ²Central Laboratory, Provincial Hospital affiliated to Shandong University, Jinan, China

³Department of Endocrinology and Metabolism, Taipei City Hospital, Ren-Ai Branch, Taipei, Taiwan

⁴Department of Dentistry, School of Dentistry, Institute of Oral Biology, National Yang-Ming University, Taipei, Taiwan

⁵Department of Education and Research, Taipei City Hospital, Taipei, Taiwan

Correspondence
should be addressed
to W-C Li or J Zhao

Email
wcli@ym.edu.tw or
jjzhao@medmail.com.cn

Abstract

AMP-activated protein kinase (AMPK) is a critical regulator of cellular and whole-body energy homeostasis. Twelve AMPK-related kinases (ARKs; BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4, and MELK) have been identified recently. These kinases show a similar structural organization, including an N-terminal catalytic domain, followed by a ubiquitin-associated domain and a C-terminal spacer sequence, which in some cases also contains a kinase-associated domain 1. Eleven of the ARKs are phosphorylated and activated by the master upstream kinase liver kinase B1. However, most of these ARKs are largely unknown, and the NUAK family seems to have different regulations and functions. This review contains a brief discussion of the NUAK family including the specific characteristics of NUAK1 and NUAK2.

Key Words

- ▶ AMP-activated protein kinase (AMPK)
- ▶ AMPK-related kinase
- ▶ NUAK family
- ▶ LKB1
- ▶ ARK5
- ▶ sucrose-non-fermenting protein kinase (SNF1)/AMPK-related kinase (SNARK)

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The AMP-activated protein kinase (AMPK) and AMPK-related kinases

Obesity and diabetes mellitus (DM) currently threaten the health of virtually every country in the world. Impaired energy balance is known as a primary constituent in the etiology of obesity and type 2 DM. AMP-activated protein kinase (AMPK), a highly conserved serine/threonine protein kinase (PK) with diverse substrates, has been known as a master sensor and regulator of energy homeostasis (Carling & Hardie 1989, Mitchelhill *et al.* 1994). AMPK functions as a heterotrimer composed of one catalytic subunit (α) and two regulatory subunits (β and γ) as its activity is regulated by increased intracellular AMP:ATP ratio in cells under metabolic stress (e.g. hypoxia, heat shock, and ischemia) (Carling & Hardie

1989, Mitchelhill *et al.* 1994, Hardie *et al.* 1998). AMPK has been reported to play a critical role in regulating glucose and cholesterol metabolism (Hardie *et al.* 1998) as well as cell proliferation, cell polarity (Williams & Brenman 2008), and tumorigenesis (Hashimoto *et al.* 2002, Kato *et al.* 2002) via its responsiveness to metabolic stress. Furthermore, AMPK activity could also be modulated by hormones and cytokines (Kahn *et al.* 2005), leading metabolism alterations that might sequentially contribute to tumor progression (Woods *et al.* 2005).

AMPK activation requires phosphorylation of threonine residue 172 in the activation loop of the catalytic domain by an upstream kinase (Stein *et al.* 2000). So far, three upstream kinases have been identified including liver kinase B1 (LKB1; so-called serine/threonine kinase 11

(STK11); Hawley *et al.* 2003), Ca^{2+} /calmodulin-dependent PK kinase β (CaMKK β ; Hawley *et al.* 2005, Woods *et al.* 2005), and transforming growth factor- β -activated kinase 1 (TAK1; Momcilovic *et al.* 2006). Among them, LKB1 is a tumor suppressor kinase linked with Peutz-Jeghers syndrome (Hemminki *et al.* 1998) and is known as the most important AMPK upstream kinase. More recently, 12 AMPK-related kinases (ARKs) have been identified and have shown great sequence homology to the catalytic domain of AMPK (Bright *et al.* 2009). With the exception of maternal embryonic leucine zipper kinase (MELK), all ARKs are specifically activated by LKB1 at a site equivalent to threonine 172 in AMPK (Lizcano *et al.* 2004). Although metabolic regulation by AMPK has been extensively studied, many of the ARKs remain largely uncharacterized. Recently, the microtubule-associated protein-regulating kinase/microtubule affinity regulating kinase (MARK) family (MARK1, MARK2, MARK3, and MARK4) has been shown to regulate cell polarity (Drewes *et al.* 1997) and the brain-specific kinase (BRSK) family (BRSK1 and BRSK2) was found to control neuronal polarity (Kishi *et al.* 2005). Pathologically, MARKs might be implicated in aberrant phosphorylation on tau protein leading to Alzheimer's disease both in animal models and human subjects (Wang *et al.* 2007). In the field of tumor biology, an important AMPK-regulated kinase novel (nua) kinase family (NUAK) has been reported to promote tumor progression and

metastatic capacity via the upregulation of cell proliferation, inhibition of p53-mediated tumor suppression, and increased matrix metalloproteinases (MMPs) in various cancer types (Hou *et al.* 2011, Chang *et al.* 2012, Chen *et al.* 2013). More recently, a key finding showing that NUAK1 (also known as ARK5) may play a role in regulating tumor proliferation and survival through metabolic alteration in hepatocarcinoma demonstrated that targeting cellular energy homeostasis could be a valuable strategy to eliminate Myc-deregulated tumor cells (Liu *et al.* 2012). As tumor and metabolic disease are two of the most common diseases globally, the physiological and pathological role of NUAK is further discussed. Overall, as illustrated in Fig. 1, different ARKs might share similar regulatory roles in the regulation of cellular physiology such as cell polarity and cell motility (Shorning & Clarke 2011). More investigations of interplays between AMPK and its associated kinases would be required to further elucidate collaborative roles of different AMPK kinases.

NUAK and SNARK: structure and expression

The NUAK family members, including NUAK1 (ARK5) and NUAK2 (SNARK), contain a ubiquitin-associated domain located next to the C-terminal of their catalytic domains (Bright *et al.* 2009), which is required for LKB1 phosphorylation and activation (Jaleel *et al.* 2006). Homology search

Figure 1

Functional interplay of ARKs. LKB1 often phosphorylates ARKs at threonine 172 (T172) of AMPK and corresponding sites of other ARKs (shown as black bars). After phosphorylation/activation, the AMPK-associated kinases could share common physiological regulation

of cell polarity or cell motility by controlling assembly/disassembly of cytoskeletal proteins. Full color version of this figure available via <http://dx.doi.org/10.1530/JME-13-0063>.

analysis of the ARK5 amino acid sequence reveals 55.0% overall homology to human SNARK (Suzuki *et al.* 2003a,b). While AMPK functions heterotrimerically, it is still unknown whether ARK5 and SNARK also act as a heterotrimer. The human ARK5 protein is composed of 661 amino acids (around 76 kDa), mouse ARK5 is predicted to contain 658 amino acids, and rat ARK5 protein is a polypeptide containing 660 amino acids (X Sun, S J Lessard, D An, H-J Koh, M F Hirshman & L J Goodyear, 2011, unpublished observations). ARK5 mRNA and protein were detected in heart, kidney, brain, liver, and skeletal muscle. Interestingly, Fisher *et al.* reported that there are two prominent ARK5 bands in skeletal muscle by western blot analysis using homemade antibody, and the lower of the two bands showed the same molecular weight as AMPK, indicating potential interaction of ARK5 with a novel AMPK family kinase (Suzuki *et al.* 2005). The expression of ARK5 is closely associated with matrix metalloproteinase 2 (MMP2), MMP9, and S100 calcium-binding protein A4 (S100A4) (Roh *et al.* 2010) and strongly correlates with a poor prognosis of multiple myeloma (Suzuki *et al.* 2005). Furthermore, ARK5 expression is also associated with the v-raf murine sarcoma viral oncogene homolog B1 (BRAF)-mediated pathway and BRAF is considered a novel indicator of progression and aggressiveness in papillary thyroid cancer (Xing 2007). ARK5 mRNA expression in colon cancer is associated with stage, and liver metastatic foci of colon cancer express very high levels of ARK5 mRNA (Kusakai *et al.* 2004a,b). In addition, the mRNAs of ARK5 and sucrose-non-fermenting PK (SNF1)/ARK (SNARK) are detected in human colorectal carcinoma cell lines DLD-1, WiDr, HCT-15, SW620, LoVo, SW480, and SW1116 (Kusakai *et al.* 2004a). Nevertheless, ARK5 is more highly expressed in differentiated C2C12 myotubes than in the undifferentiated myoblasts implying a versatile role of NAUK/ARK proteins in different cell types (Niesler *et al.* 2007).

SNARK, the fourth member of the AMPK catalytic subunit family, was originally identified as a u.v. B-induced gene in keratinocytes (Rosen *et al.* 1995). Rat *Snark* cDNA is 2929 bp long and encodes 630 amino acids (Lefebvre *et al.* 2001). The human *SNARK* gene is located at chromosome 1q32.1 encoding a 628 amino acid protein with an estimated molecular weight of 69 kDa (Lefebvre *et al.* 2001, Suzuki *et al.* 2003c). SNARK is translated into a single 76 kDa protein, while western blot of another 80 kDa isoform could be detected in baby hamster kidney cells (Lefebvre *et al.* 2001). Recently, a homology search analysis showed that SNARK has 45.9, 41.2, 41.3, and 55.3% homology to AMPK- α 1, AMPK- α 2, MELK, and ARK5 respectively (Suzuki *et al.* 2003a,b). Different from the

cytoplasmic expression of AMPK α , SNARK is predominantly localized in the nucleus even when it is activated by metabolic stimuli, and it can affect gene expression profiles and functions as a transcriptional modulator in the nucleus in response to stresses (Kuga *et al.* 2008). In rats, SNARK has been detected in the kidney, thymus, spleen, and stomach, with higher levels found in the skin, testis, uterus, and ovary and highest levels in the adrenal and brain tissues (Lefebvre & Rosen 2005). High expression of SNARK protein was also found in HL1 cardiomyocytes (X Sun & J Zhou, 2013, unpublished observations), as well as in the skeletal muscle and heart tissues of mice (Lefebvre & Rosen 2005). Interestingly, the more oxidative muscles (gastrocnemius and soleus), as well as the heart, appeared to express two forms of SNARK (Koh *et al.* 2010). A recent study reported that SNARK expression is increased in skeletal muscle of human subjects with obesity as well as in response to with metabolic stressors, but not in type 2 DM (Rune *et al.* 2009).

The function and regulation of ARK5

Similar to other AMPK family members, ARK5 contains a highly conserved active T loop, suggesting the presence of an upstream kinase that phosphorylates the threonine residue in the catalytic domain. The phosphorylation of ARK5 at threonine 211 by the LKB1 and serine 600 by Akt kinase has been shown to activate its kinase activity (Suzuki *et al.* 2003b, 2006). Like most AMPK-regulated kinases, ARK5 activity is increased 10- to 20-fold by phosphorylation of its T loop threonine by LKB1 (Lizcano *et al.* 2004). A recent study showed that basal and contraction-stimulated ARK5/SNARK immune complex activity is significantly decreased in skeletal muscles from muscle-specific *Lkb1* knockout mice using an antibody that does not differentiate between the two proteins (Koh *et al.* 2010). This suggested that SNARK and/or ARK5 could be essential regulators of LKB1 in skeletal muscle. Recently, Matrigel invasion assays demonstrated that both overexpressed and endogenous ARK5 showed strong Akt-mediated activity (Suzuki *et al.* 2004). Importantly, ARK5 is the only Akt-regulated AMPK family member to date.

ARK5 is stimulated *in vitro* by AMP and phosphorylates SAMS peptide, which is a common synthetic substrate for AMPK family members (Suzuki *et al.* 2003a,b, 2006). ARK5 is activated in an event downstream of growth factors, such as insulin or insulin-like growth factor 1 (IGF1), which bind to corresponding receptors to activate cell signaling by phosphorylation of insulin receptor substrates (Suzuki *et al.* 2003a,b). Moreover, the phosphorylation level of

ARK5 T loop threonine is increased by contraction or exposure to AICAR in rat skeletal muscle (Fisher *et al.* 2005), although overexpression of WT ARK5 and mutant ARK5 (Thr²¹¹ to Ala) did not alter basal and contraction-stimulated glucose uptake (Koh *et al.* 2010). It is noteworthy that NUA1/ARK5 could suppress glucose uptake through negative regulation of insulin signaling in oxidative muscle depicting delicate NUA1/ARK5-mediated regulatory mechanisms in differential physiological conditions (Inazuka *et al.* 2012).

ARK5 is closely associated with human malignancy (Kusakai *et al.* 2004a). ARK5 induces tumor cell survival during nutrient starvation and suppresses cell death caused by glucose starvation, TRAIL, and TNF α , but not by u.v. irradiation, camptothecin, or doxorubicin (Suzuki *et al.* 2003a,b). Recently, ARK5 was discovered as a major factor in Akt-dependent cancer cell survival and migration activity through activation of membrane-type 1 MMP (MT1-MMPs) *in vitro*, while the detailed mechanisms remain to be explored (Kusakai *et al.* 2004a). It is possible that Akt is activated by threonine 308 and serine 473 phosphorylation and then the activated Akt inhibits apoptosis and stimulates invasion activity by phosphorylating the downstream substrate ARK5 at Ser600 (Kusakai *et al.* 2004a, Lefebvre & Rosen 2005, Woods *et al.* 2005). Overexpression of ARK5 confers tolerance to glucose starvation, which is a stress that leads to a decrease in ATP and an increase in AMP. Therefore, it is probably that insufficient blood supply could lead to hypoxia sequentially causing activation of ARK5 through both Akt activation and increased AMP. This may then result in tolerance to nutrient starvation and upregulation of MT1-MMP production, thereby leading to MMP2 and MMP9 activation and stimulation of tumor invasion and metastasis (Koh *et al.* 2010). In brief, the Akt/ARK5 pathway is probably a new signaling pathway for the induction of the cell survival that is closely related to tumorigenesis.

Recently, the nuclear DBF-related kinase 2 (NDR2) has also been found to phosphorylate threonine 211 on the active T loop of ARK5 and could be activated upon IGF1 treatment (Suzuki *et al.* 2006). Furthermore, ARK5 is transcriptionally regulated by c-Maf through MAF-recognition element (MARE) and may in part mediate the aggressive phenotype associated with c-MAF and MAFB-expressing myelomas (Fisher *et al.* 2005, Niesler *et al.* 2007). Another study showed that ARK5 could inhibit, by phosphorylation of Fas-associated death domain-like interleukin 1 β -converting enzyme-inhibitory protein (FLIP), caspase-8 and caspase-6 in glucose-

deprived tumor cells (Woods *et al.* 2005) as further amino acid sequence analysis of caspase-6 protein revealed two putative sites of phosphorylation by ARK5: Ser80 and Ser257 (Suzuki *et al.* 2004). ARK5 also regulates ploidy and senescence. Decreased ARK5 can prevent cells from the state of aneuploidies and enhance their replicative lifespan, while increased ARK5 induces gross aneuploidies and senescence (Humbert *et al.* 2010). Taken together, the ARK5 pathway might play an important role in cancer progression. As ARK5 may be a novel tumor progression-associated factor, it is expected that inactivation of ARK5 might be a novel therapeutic approach in human tumors with a poor prognosis. Overall, ARK5-associated molecular regulatory inputs and sequential downstream cellular outputs are illustrated in Fig. 2A.

The function and regulation of SNARK

Another ARK, SNARK, is capable of autophosphorylation, and immunoprecipitated SNARK exhibited phosphotransferase activity with the synthetic peptide SAMS as a kinase substrate (Lefebvre *et al.* 2001). SNARK could interact with ubiquitin-specific protease 9, X chromosome (USP9X), a deubiquitinating enzyme that catalyzes the deubiquitination of the kinase while non-USP9X binding mutants of SNARK are inactive (Al-Hakim *et al.* 2008). SNARK is activated in a cell-type-specific manner by a variety of stimuli including hyperosmotic stress, DNA damage and oxidative stress, AMP, 5-amino-4-imidazolecarboxamide riboside (AICA riboside), and nutrient deprivation including glucose and glutamine deficiency (Lefebvre *et al.* 2001, Lefebvre & Rosen 2005). For instance, glucose deprivation increases SNARK activity threefold in BHK fibroblasts (Lefebvre *et al.* 2001) and enhances cell survival in HepG2 cells (Suzuki *et al.* 2003c). Furthermore, SNARK activity is increased by contraction in mouse-isolated extensor digitorum longus muscle and by treadmill exercise in both mouse and human skeletal muscle (Koh *et al.* 2010). By contrast, overexpression of mutant *Snark* impaired contraction-stimulated glucose uptake in mouse tibialis anterior muscle and knockdown of *Snark* impairs sorbitol-induced glucose uptake in C2C12 cells (Koh *et al.* 2010).

Several aspects of SNARK function and regulation are highly compatible to AMPK (Egan & Zierath 2009). For example, both SNARK and AMPK are AMP responsive and could be activated by treatments known to increase the AMP:ATP ratio including glucose deprivation and chemical ATP production (Lefebvre *et al.* 2001, Lefebvre & Rosen 2005, Kuga *et al.* 2008). In addition, LKB1

Figure 2

Cellular and molecular regulatory circuits for (A) NUA1 and (B) NUA2. All abbreviations are described in text. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-13-0063>.

phosphorylated SNARK at Thr208, a residue equivalent in position to Thr172 within the activation loop of AMPK α 2 (Lizcano *et al.* 2004). However, there are still some variances between SNARK and AMPK. For example, either AICAR or phenformin failed to activate SNARK in mouse embryonic fibroblasts (Lefebvre & Rosen 2005, Egan & Zierath 2009) while SNARK silencing by siRNA does not influence basal and insulin-stimulated glucose uptake, glucose incorporation into glycogen, or AICAR-stimulated lipid oxidation (Rune *et al.* 2009).

LKB1 is a rate-limiting enzyme to activate SNARK as it could stimulate SNARK enzymatic activity by as great as

50-fold, implying that SNARK may mediate one or more physiological effects of LKB1 (Lefebvre & Rosen 2005). Interestingly, SNARK T-loop peptide is a better LKB1 substrate than AMPK α 1 peptide, suggesting the differential kinase binding affinity of LKB1 with AMPK-related kinases (Lizcano *et al.* 2004). Furthermore, activities of endogenous SNARK are markedly reduced in LKB1-deficient cells (Lizcano *et al.* 2004) as the data cannot rule out the possibility that other kinases are regulating the activity of the ARKs *in vivo* in addition to LKB1.

SNARK activation due to glucose deprivation is shown to induce acute cell-cell dissociation, which correlated

well with the detection of G-actin, in a hepatoma cell line, suggesting that SNARK may play a role in cell motility associated with carcinogenesis (Lefebvre *et al.* 2001, Suzuki *et al.* 2003c). Moreover, the putative catalytic domain of SNARK is necessary for the cell–cell detachment and phosphorylation of focal adhesion kinase and PKC, which are dramatically increased by glucose starvation in HepG2 cells, and is markedly suppressed by SNARK (Suzuki *et al.* 2003c). Recently, SNARK was reported as a downstream molecule of EBV latent membrane protein 1, which is associated with resistance to cancer cell death (Kim *et al.* 2008). In addition, SNARK has been shown to have anti-apoptotic properties, protecting cells from TNF-related apoptosis inducing ligand-induced apoptosis. Knockdown experiments suggested that SNARK is also involved in the CD95-induced motility and invasiveness in MCF7-FB cells (Legembre *et al.* 2004). Overall, SNARK could be an important regulator for cancer cell viability, migration, and metastatic capacity.

Until now, only a single substrate of SNARK, myosin phosphatase target subunit (MYPT1), has been identified with an *in vitro* kinase assay (Yamamoto *et al.* 2008). MYPT1 is a regulatory subunit of myosin phosphatase catalyzing dephosphorylation of myosin light chain. Myosin light chain regulates many cellular functions including smooth muscle contraction (Ito *et al.* 2004). SNARK phosphorylates MYPT1 at a different site other than published Rho-kinase (ROCK) phosphorylation sites, suggesting an alternative unknown SNARK-mediated MYPT-1 phosphorylation regulatory mechanism, which remains to be investigated (Yamamoto *et al.* 2008).

Recently, it was reported that homozygous *Snark*^{−/−} knockout mice have a high incidence of embryonic lethality, whereas the heterozygous *Snark*^{+/-} knockout mice develop mature-onset obesity, hepatic steatosis, altered serum lipid profiles, hyperinsulinemia, hyperglycemia, impaired glucose tolerance, and enhanced chemical carcinogen-induced neoplastic and preneoplastic colorectal lesions (Tsuchihara *et al.* 2008). Most of these symptoms are similar to those of human type 2 DM accompanied by obesity. In addition, fat synthesis and deposition are enhanced, accompanied by a reduction in total body temperature and daily energy expenditure, as assessed by oxygen uptake, in *Snark*^{+/-} mice (Tsuchihara *et al.* 2008). Habitual food intake between *Snark*^{+/-} and WT animals in the sedentary state does not differ (Ichinoseki-Sekine *et al.* 2009). A recent study reports that contraction-stimulated glucose uptake is impaired in skeletal muscles from *Snark*^{+/-} mice, while insulin-stimulated glucose transport and the profile of the

proliferating cell population is not altered (Koh *et al.* 2010). This defect is not due to altered contraction force and probably is associated with blunted AS160/TBC1D1 PAS (phospho (Ser/Thr) Akt substrate) phosphorylation (Koh *et al.* 2010). This finding could be linked to the role of SNARK in cancer formation, suggesting that SNARK deficiency could take part in tumor formation but not cell proliferation *in vivo* (Tsuchihara *et al.* 2008). Alternatively, ARK5 and SNARK can phosphorylate large tumor suppressor 1 (LATS1) while AMPK α 2 cannot, although the mechanism of SNARK regulation seems to be different from ARK5. ARK5 is able to regulate LATS1 protein levels directly through phosphorylation at Ser464, while SNARK might phosphorylate full-length LATS1 protein or LATS1 peptide and decrease LATS1 levels (Humbert *et al.* 2010). In summary, the above findings suggest that SNARK plays a critical role in the modulation of whole-body bioenergetics, although further characterization of this phenotype is still required. Figure 2B schematically summarizes the environmental stimuli and SNARK-mediated molecular and physiological events.

Conclusion

The NUA family is essential in malignancy and energy metabolism. Further work is needed to elucidate the regulatory mechanisms and functions of NUA family proteins. Although the functions of the NUA family are not fully understood, ARK5 and SNARK are expected to be potential therapeutic targets for treatment of human cancers and metabolic disorders, such as obesity and DM.

Declaration of interest

The authors do not have and have not had any actual or potential conflict of interest within 3 years of beginning the work submitted. All authors agreed to submit the work to *Journal of Molecular Endocrinology*, and the work has not been submitted to another journal.

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