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A hypothetical MEK1-MIP1-SMEK multiprotein signaling complex may function in *Dictyostelium* and mammalian cells

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ABSTRACT In a previous study, we characterized *Dictyostelium* SUMO targeted ubiquitin ligase (StUbL) MIP1 that associates with protein kinase MEK1 and targets SUMOylated MEK1 to ubiquitination (Sobko *et al.,* 2002). These modifications happen in response to activation of MEK1 by the chemoattractant cAMP. Second site genetic suppressor of mek1- null phenotype (SMEK) was also identified in *Dictyostelium*. MEK1 and SMEK belong to the same linear pathway, in which MEK1 negatively regulates SMEK, which then negatively regulates chemotaxis and aggregation. RNF4 is mammalian homologue of MIP. RNF4 interacts with hSMEK2, the human homologue of *Dictyostelium* SMEK. We propose the existence of an evolutionarily conserved MEK1-SMEK signaling complex that upon MEK1 activation and SUMOylation, recruits ubiqutin ligase MIP1/RNF4, which, in turn, ubiquitinates SMEK and targets this protein for proteasomal degradation. This could be a mechanism for negative regulation of SMEK by MEK1 signaling.

KEY WORDS: *RNF4, SUMO-targeted ubiquitin ligase, suppressor of MEK (SMEK), ubiquitination, cell migration*

Cell motility and primitive mode of amoeboid migration are fundamental and ancient cellular behaviors that contribute to multicellular development, inflammation, immune responses, cancer metastasis and are conserved between mammals and non-mammalian model organisms, such as *Dictyostelium discoideum* (Stuelten *et al.*, 2018). Dictyostelium presents a simple model to study directed cell migration (chemotaxis). The evolutionary conservation of canonic signaling pathway modules, accessible genetics (now including knock-outs, knock-ins, expression of engineered sequences, RNAi and CRISPR perturbations) (Sekine *et al.,* 2018) and amenability to live imaging make Dictyostelium an important model to examine basic molecular mechanisms that govern chemotaxis. This organism permits direct observation of cells moving in complex native environment and allows large-scale genetic and pharmacological screening, as well as extensive biochemical and cell biology studies. Among genetically dissected pathways, MAP kinase pathways are central in control of aggregation and chemotaxis. The fundamental architecture of MAP kinase pathway is conserved between Dictyostelium and other eukaryotes, and this presents researchers with the opportunity to investigate conserved functions and common components, including protein kinases, kinase substrates, molecular scaffolds and regulatory proteins. Significant insights into functions of MAP kinase pathway were obtained using other genetically tractable model organisms, such as S. cerevisiae, C. elegans, and D. melanogaster. This cross-species data allows now to make comparisons between the phenotypes of these models, in which specific components of the pathway were perturbated (Shilo, 2014). It is also noteworthy, that ubiquitination machinery relevant for our discussion below has been extensively studied in Dictyostelium (Pergolizzi *et al.,* 2019), and our study provides one important paradigm of how ubiquitination regulates protein kinase signaling and cellular functions (Sobko *et al.,* 2002).

SUMO-targeted Ubiquitin Ligases (StUbLs) that recruit SUMOylated substrates to ubiquitination machinery have been characterized in fission and budding yeast, *Drosophila* and mammals (Sriramachandran and Dohmen, 2014), (Abed *et al.,* 2018). In each system, specific individual substrates of StUbLs and their cellular functions were identified. In our previous study (Sobko *et al.,* 2002), we characterized *Dictyostelium* StUbL MIP1 that associates with protein kinase MEK1 and targets SUMOylated MEK1 to ubiquitination. These modifications happen in response to activation of MEK1 by chemoattractant cAMP. Another study from Firtel lab characterized SMEK – second site genetic suppressor of mek1- null

Abbreviations used in this paper: PTM, post-translational modification; SIM, SUMO interactive motif; SMEK, suppressor of MEK; StUbL, SUMO targeted ubiquitin ligase.

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phenotype (Mendoza *et al.,* 2005, Mendoza *et al.,* 2007). Deletion of mek1 gene results in the phenotype, in which cells fail to aggregate properly and form very small aggregates, due to severe chemotaxis defect. Suppressor phenotype of SMEK implies that aggregation/chemotaxis defect of mek1 null cells is rescued upon deletion of smek gene in mek1- null cells. According to Mendoza *et al.*, the analysis of smek phenotype shows, that not all effects of SMEK occur via MEK1 signaling. Nevertheless, at least in part, MEK1 sends the signal to SMEK, which, in turn, negatively affects chemotaxis and aggregation. Therefore, we propose the following scenario: MEK1 and SMEK belong to the same linear pathway, in which MEK1 negatively regulates SMEK, which then negatively regulates chemotaxis and aggregation.

Curiously, human SMEK1 and SMEK2 encode evolutionarily conserved isoforms of regulatory subunits of serine/threonine-protein phosphatase 4, that among other functions, were implicated in control of cell migration (Martin-Granados *et al.,* 2008, Gingras *et al.,* 2005), (see also entries for SMEK1 and SMEK2 in GeneCards database for more information), (Rebhan *et al.,* 1998).

In *C.elegans*, SMEK homologue was shown to function downstream FOXO transcription factor DAF-16 in canonical Insulin/IGF-1 signaling pathway, regulating longevity and stress responses (Wolff *et al.,* 2006).

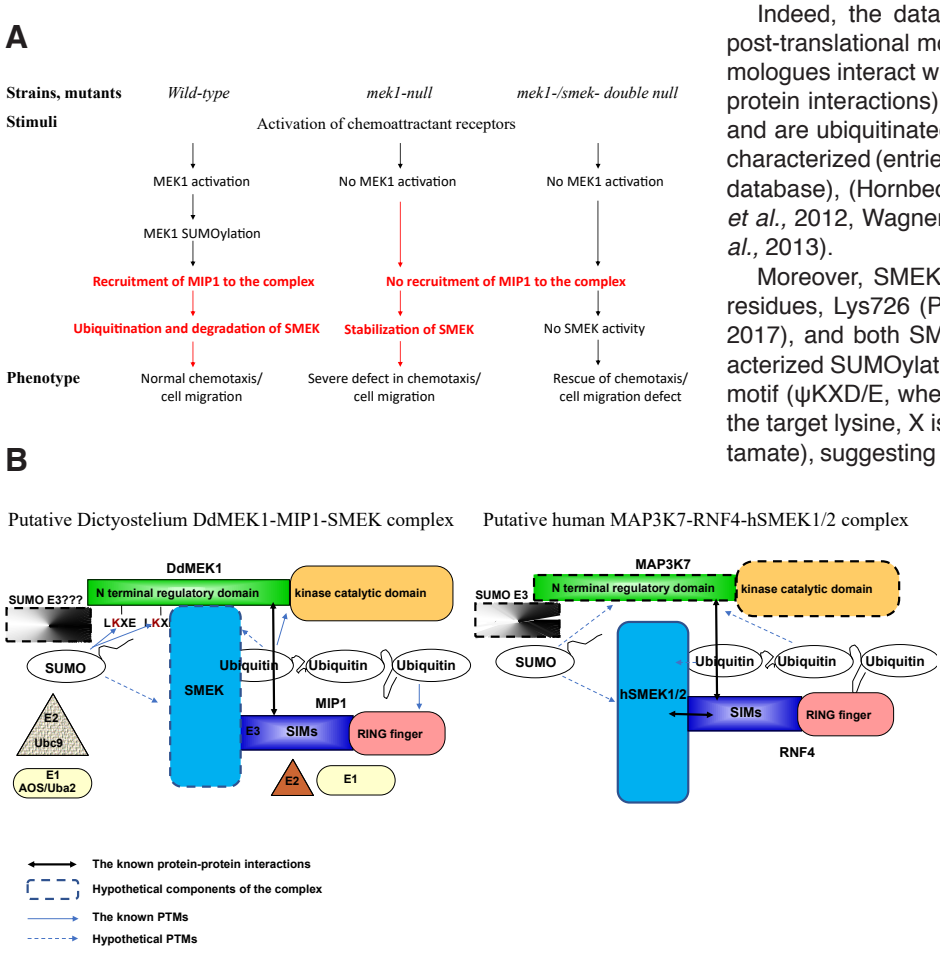

Fig. 1. The MEK1-MIP1-SMEK signaling complex. (A) *Regulation of chemotaxis by putative MEK1-MIP1-SMEK signaling complex. The known and the hypothetical connections (the latter in red text).* **(B)** *The known and hypothetical interactions within multiprotein signaling complexes.*

MIP1 is *Dictyostelium* RING Finger protein, which belongs to recently discovered evolutionarily conserved group of StUbL proteins, that contain also SUMO Interactive Motif (SIM) and drive SUMOylated proteins to ubiquitination and subsequent proteasomal degradation (Sobko *et al.,* 2002, Sun *et al.,* 2007, Geoffroy & Hay, 2009).

RNF4 is mammalian homologue of MIP. It also contains SIM and RING Finger domains and it is most likely functions as StUbL. Recently, RNF4-interacting proteins were systematically identified in high throughput proteomics/mass spectrometry study (Kumar *et al.,* 2017). Intriguingly, the data of this study shows that RNF4 interacts with human homologue of *Dictyostelium* SMEK – hSMEK2. This raises the possibility, that such complex is conserved in evolution, and exists in both human and *Dictytostelium* cells. If in human cells RNF4 interacts with hSMEK2, then, MIP1 possibly interacts with SMEK in *Dictyostelium*. We propose that MEK1 and SMEK interact not only genetically, but also physically. Moreover, the mechanism of negative regulation of SMEK by MEK1 could be based on the existence of MEK1-SMEK complex that upon MEK1 activation and SUMOylation, recruits StUbL MIP1. It is possible, that MIP1 ubiquitinates SMEK and targets this protein for proteasomal degradation. This could be a basis for negative regulation of SMEK by MEK1 signaling.

Indeed, the data of high-throughput proteomics analysis of post-translational modifications (PTMs) indicates that SMEK homologues interact with each other (BioGRID database of protein-protein interactions), (Oughtred *et al.,* 2019, Huttlin *et al.,* 2017) and are ubiquitinated on multiple lysine residues that have been characterized (entries for SMEK1 and SMEK2 in PhosphoSitePlus database), (Hornbeck *et al.,* 2015, Akimov *et al.,* 2018, Povlsen *et al.,* 2012, Wagner *et al.,* 2011, Mertins *et al.,* 2013, Udeshi *et al.,* 2013).

Moreover, SMEK2 is also SUMOylated on one of the lysine residues, Lys726 (PhosphoSitePlus database), (Lumpkin *et al.,* 2017), and both SMEK1 and SMEK2 possess putative uncharacterized SUMOylation sites, that fit the consensus SUMOylation motif (ψKXD/E, where ψ is a large hydrophobic amino acid, K is the target lysine, X is any amino acid and D/E is aspartate or glutamate), suggesting that MEK-SMEK complex might be subject to SUMOylation that serves as a signal to bind StUbl MIP1/RNF4, which likely targets the complex to subsequent ubiquitination and proteasomal degradation.

This data needs to be further validated under relevant physiological conditions and stimuli, such as exposure to the chemoattractant. In our future studies, using Dictyostelium chemotaxis and aggregation, as experimental system, we would like to apply immune affinity purification and co-immunoprecipitation of tagged expressed proteins to prove that, MEK1, MIP and SMEK indeed form multi-protein complex. We would like to verify whether SMEK is SUMOylated and ubiquitinated upon chemoattractant stimulation. It will be curious to check the dynamic composition of SMEK-MIP1 complexes over the time course after chemoattractant stimulation. It will be

TABLE 1

REPORTED COMPONENTS OF PUTATIVE MEK1-MIP1/RNF4-SMEK COMPLEX: KNOWN HOMOLOGUES IN OTHER ORGANISMS

Gene/Protein	Homology, similarity of amino acid sequence to Dictyostelium counterpart	Reported protein-protein and genetic interactions	References
Dictyostelium DdMEK1	-	MIP1 (yeast two-hybrid; co-IP)	Sobko et al, 2002
Dictyostelium MIP1	-	DdMEK1 (yeast two-hybrid; co-IP)	Sobko et al, 2002
Dictyostelium SMEK	-	DdMEK1 (genetic interactions – second site suppressor)	Mendoza et al, 2005, 2007
Human RNF4	Homologue of MIP1 (Identical positions 47; similar positions 78)	hSMEK2 (IP-Mass spectrometry) MAP3K7 (reciprocal IP-Mass spectrometry)	Kumar et al, 2017 BioGRID database
Human hSMEK1	Homologue of SMEK (Identical positions 252; similar positions 314)	hSMEK2 (reciprocal IP-Mass spectrometry)	BioGRID database
Human hSMEK2	Homologue of SMEK (Identical positions 247; similar positions 319)	hSMEK1 (reciprocal IP-Mass spectrometry)	BioGRID database
Human MAP3K7	MAP kinase kinase kinase	RNF4 (reciprocal IP-Mass spectrometry)	BioGRID database
C. elegans SMK-1	Homologue of SMEK (Identical positions 222; similar positions 370)	Genetic interactions with FOXO transcription factor DAF-16 (Insulin/IGF-1 signaling pathway)	Wolff et al, 2006

Reported protein-protein and genetic interactions. Amino acid sequence identity and similarity were determined with UniProt (https://www.uniprot.org) Align tool.

instrumental to compare steady-state levels of SMEK ubiquitination under the same conditions in cells either overexpressing MIP1 or possessing mip1 gene deletion.

It will be also important to validate RNF4 – hSMEK2 interactions and existence of the complex in human cells (using suitable cell lines with high expression levels of these proteins). One may hypothesize that RNF4-SMEK complex also interacts with the protein kinase of one of mammalian MAP kinase pathways. Activation of this kinase would be expected to trigger SUMOylation, ubiquitination and proteasomal degradation of SMEK and possibly other components of the complex. For example, we would need to establish whether putative RNF4-hSMEK2 complex also contains mitogen-activated protein kinase kinase kinase, MAP3K7, which was found reciprocally as either "bait" or "pray" in mass spectrometry/proteomics study of RNF4-interacting proteins (BioGRID entries for RNF4 and MAP3K7), (Tan *et al.,* 2015). MAP3K7 is known to be ubiquitinated in response to cytokine activation and signaling. Does MAP3K7 ubiquitination require RNF4, as a component of StUbL? Is MAP3K7 also SUMOylated? Is SUMOylation a prerequisite for ubiquitination? All these questions await further experimentation. Ultimately, we would like to apply systems biology analysis of protein-protein interactions and molecular pathways to identify other putative components of these conserved multi-protein signaling complexes and explore their functions.

The availability of data from global studies of protein-protein interactions and PTMs of protein kinases and other signaling proteins now makes it possible to predict and validate functional connections between the kinases, their putative substrates, modulators and other proteins (Sobko, 2006).

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Conflict of interest

The author has no a conflict of interest.

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