The oxysterol-binding protein superfamily: new concepts and old proteins

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Abstract
The Kes1 OSBP (oxysterol-binding protein) is a key regulator of membrane trafficking through the TGN (trans-Golgi network) and endosomal membranes. We demonstrated recently that Kes1 acts as a sterol-regulated rheostat for TGN/endosomal phosphatidylinositol 4-phosphate signalling. Kes1 utilizes its dual lipid-binding activities to integrate endosomal lipid metabolism with TORC1 (target of rapamycin complex 1)-dependent proliferative pathways and transcriptional control of nutrient signalling.

Introduction
Golgi and endosomal systems are highly dynamic organelles that undergo organized renewal and maturation [1]. These organelles are central sites of intracellular signalling and membrane sorting. The co-ordination of signalling and trafficking functions of the Golgi/endosomes is not understood, but could potentially act through a lipid signalling interface. Lipid-transfer proteins help to coordinate lipid metabolism with core protein components of the membrane trafficking machinery through the TGN (trans-Golgi network) and endosomal membranes [2–5]. Specifically, the yeast PtdIns/PtdCho (phosphatidylcholine)-transfer protein Sec14 and the OSBP (oxysterol-binding protein) family member Kes1/Osh4 regulate trafficking through the TGN/endosomal system [2,3,6]. Sec14 acts as a coincidence sensor that couples PtdCho metabolism with production of PtdIns4P [7,8]. Kes1 dampens Sec14-regulated PtdIns4P signalling in the TGN/endosomes [9,10]. It remains unclear why cells utilize an opposing pair of lipid transfer proteins in the TGN/endosomes. In our studies, we have demonstrated that Kes1 uses its dual lipid-binding activities to integrate multiple aspects of lipid metabolism in late stages of the secretory pathway with TORC1 (target of rapamycin complex 1) and nutrient signalling in yeast [11]. In the present paper, we review the primary findings of that work.

Effects of sterol and lipid binding on Kes1 function
Kes1 is a PtdIns4P- and sterol-binding protein [9,12], and Kes1 localization to the TGN/endosomal membranes requires a pool of PtdIns4P derived from the phosphoinositide 4-kinase Pik1 [9,10]. However, how Kes1 co-ordinates its dual lipid-binding activities was previously unknown. Several studies suggest that Kes1 acts as a diffusible carrier for sterols at sites of membrane apposition where the diffusion space between sterol ‘donor’ and ‘acceptor’ membranes is limited [12–15]. To better study the effects of PtdIns4P and sterol binding on Kes1 function, alleles were generated to specifically inhibit binding of either lipid or to ablate binding to both. The kes1E triple mutant (kes1E) is defective for PtdIns4P binding, but not sterol binding [11]. This mutant is biologically inactive, in part because it cannot associate with the TGN/endosomal membranes [9]. Sterol-binding mutants of Kes1 were also generated [11,12,16–18]. The kes1Y97F and kes1T185V mutants prevent sterol binding by disrupting a critical network of ordered water molecules deep within the sterol-binding pocket. Kes1 is non-essential in vegetative cells; however, it inhibits cell growth when overexpressed in yeast cells [3,11,16]. Our studies show that expression of Kes1 and the sterol-binding mutants kes1Y97F and kes1T185V (kes1Y97F is reported to be non-functional [12]) inhibit cell growth, whereas the PtdIns4P-binding mutant kes1E has no effect on cell growth [11]. In fact, kes1Y97F and kes1T185V are even more toxic to yeast than is Kes1 itself. These findings contradict reports that ablation of sterol-binding activity results in simple inactivation of Kes1 [12,13].

That Kes1 sterol-binding mutants represent hyperactive forms of Kes1 is of interest in that kes1Y97F shows enhanced association with the TGN/endosomal membranes relative to Kes1 itself [11]. As with Kes1, the kes1E PtdIns4P-binding and TGN/endosome-targeting defects are sufficient to disrupt kes1Y97F super-recruitment to endosomal membranes and neutralize kes1Y97F-mediated growth inhibition. Together, these findings are inconsistent with models describing a physiologically significant role for Kes1 in non-vesicular sterol transfer. Rather, it is more likely that Kes1 functions as a sterol sensor on the TGN/endosomal membranes (see below).
Kes1 super-recruitment to the TGN/endosomal membrane impairs vesicular trafficking

Kes1 regulates Golgi-derived vesicular transport by clamping Pik1-generated Golgi PtdIns4P [9–11]. We and others have shown that increased levels of Kes1 impair trafficking through the TGN/endosomal system. Specifically, trafficking of CPY (carboxypeptidase Y), the v-SNARE (vesicular soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) Snc1, and the bulk endocytic tracer FM4-64 is perturbed by ectopic expression of Kes1 and the sterol-binding mutants kes1Y97F and kes1T185V [3,11,16]. We found that Kes1 sterol-binding mutants had greater inhibitory effects on CPY trafficking than did Kes1 itself [11].

These data suggest that Kes1 acts as a rheostat for TGN/endosomal PtdIns4P signalling whose activity depends on PtdIns4P binding to Kes1. Sterol binding to Kes1 promotes Kes1 release from PtdIns4P in the TGN/endosomal membrane (Figure 1). The dual-binding activity of Kes1 allows it to act as a sterol-regulated ‘brake’ on PtdIns4P-dependent trafficking through the endosome. This is demonstrated by increased association of the sterol-binding mutant kes1Y97F to the TGN/endosomal membrane, ultimately resulting in inhibition of cell growth and diminished trafficking through TGN/endosomes. These data suggest that Kes1 is a sterol sensor in the TGN/endosomal system, rather than a sterol-transfer protein.

Kes1 and nutrient signalling

Kes1 intoxication causes growth inhibition and defects in trafficking without significant loss of viability. In the following sections, we summarize the effects of Kes1/kes1Y97F overexpression on nutrient signalling.

Effects of Kes1 overexpression on autophagy

Although yeast expressing Kes1/kes1Y97F exhibit membrane trafficking defects through the TGN/endosomal system, thin-section electron microscopy analyses did not find the expected accumulation of aberrant toroid-shaped membrane systems, called Berkeley bodies, that are indicative of cargo-engorged secretory compartments [19,20]. Instead, we observed intravacuolar vesicles in Kes1- and kes1Y97F-intoxicated cells, suggesting that autophagic processes are induced in these cells despite their growth in nutrient-sufficient medium [11]. This was further confirmed using both an in vivo reporter assay for monitoring the autophagic-dependent processing of the otherwise cytosolic Pho8Δ60 alkaline phosphatase, as well as visualizing the mobilization of Atg18 from the cytosol to pre-autophagosomal compartments in Kes1- and kes1Y97F-overbearing cells [11]. Interestingly, an earlier preliminary study claimed that Kes1 inhibits autophagy [16], which we find is clearly not the case.

Kes1 and GAAC (general amino acid control) pathway

The onset of precocious autophagic responses diagnoses nutrient-sensing defects in cells with elevated Kes1/kes1Y97F. Metabolomic profiling found a decrease in intracellular amino acid pools in Kes1/kes1Y97F yeast [11]. The most prominent decreases were of arginine, asparagine, aspartate, glutamate, glutamine, threonine and tryptophan. We found that spiking the medium with a concentrated NEQR (asparagine/glutamate/glutamine/arginine) cocktail rescued growth of Kes1/kes1Y97F-arrested cells. However, the NEQR cocktail did not rescue defects in TGN/endosomal trafficking, suggesting that Kes1 intoxication primarily results from nutrient-sensing defects rather than the membrane trafficking defects alone.

Supplementation with asparagine was found to be necessary, although not sufficient, to alleviate Kes1-dependent growth defects. We infer from this asparagine requirement that ammonium (NH₄⁺) starvation is the core nutritional insult to Kes1-intoxicated cells. This is supported by the observations that (i) the expression of genes encoding the ammonium permeases MEP1 and MEP2 is greatly diminished in Kes1/kes1Y97F-arrested cells, and (ii) ASP1 deletion prevents the NEQR cocktail from rescuing growth inhibition of Kes1-intoxicated cells. ASP1 encodes an asparaginase which produces glutamine by transferring an amino group from asparagine to glutamate. Glutamine acts as a biosynthetic NH₄⁺ pool and has other roles in amino acid uptake, TOR (target of rapamycin) activation and maintenance of mitochondrial integrity [21]. Interestingly, supplementation of glutamine alone cannot rescue the Kes1/kes1Y97F growth defect, suggesting that asparagine may have another role. It was shown previously that NH₄⁺ starvation blocks translation of GCN4 mRNA in an eIF2α (eukaryotic initiation factor 2α)-independent manner, attenuating the GAAC pathway [22]. Kes1 intoxication...
recapitulates this effect. The mammalian GCN (general control non-derepressible) 2/ATF4 (activating transcription factor 4) (homologous with yeast Gcn2/Gcn4) pathway specifically requires asparagine for tumour cell survival [23].

We also found defects in TORC1 signalling in Kes1/kes1Y97F cells. Amino acids promote TORC1 signalling by modulating activity of the Rag GTPases Gtr1 and Gtr2 [24–27]. Neither casamino acids nor the NEQR cocktail can rescue growth of Kes1-intoxicated gtr1Δa or gtr2Δa cells [11]. This suggests that the NEQR cocktail rescues growth of Kes1-intoxicated cells by restoring Gtr-mediated activation of TORC1.

The GAAC pathway is activated by Gcn2-mediated phosphorylation of eIF2α which decreases eIF2α activity and specifically promotes translation of the Gcn4 transcription factor open reading frame [28,29]. Kes1/kes1Y97F cells exhibit enhanced levels of phospho-eIF2α, but do not induce transcription of the Gcn4-dependent target genes HIS4 and ARGI [11,30]. We found that induction of the GAAC pathway in Kes1/kes1Y97F cells. The ChIP data suggest that yet another level of control must exist downstream of pre-initiation complex assembly to account for inhibition of ARGI transcription in response to dual 3-AT and PHS treatment of cells.

The identities of the nuclear effectors remain unknown. The catalytic subunit of the CDK8 module (Srb10) may be activated by sphingolipids at specific (Gcn4-dependent?) enhancers. Alternatively, epigenetic effects may also be involved. Recently, mammalian HDACs (histone deacetylases) were shown to be inhibited by sphingosine 1-phosphate [32]. Perhaps yeast HDACs or histone acetyltransferases are direct targets of sphingolipid-mediated regulation in this Kes1-dependent signalling pathway.

Future perspectives

We have identified a novel signal transduction pathway that is relayed from distal compartments of the secretory pathway to the nucleus where the OSBP homologue Kes1 inhibits Gcn4 activity by coupling lipid metabolism with nutrient signalling in yeast (Figure 2). Collectively, these events down-regulate TOR signalling and subsequently halt cell-cycle progression. The executioners of this pathway are highly conserved. In mammals, Kes1-like ORPs (OSBP-related proteins) are most highly expressed in terminally differentiated non-mitotic tissues, such as skeletal muscle, heart and brain [33]. We speculate that this modality helps to guide cell entry into post-mitotic states or maintains post-mitotic cell physiology. A model where Kes1 co-ordinates Golgi/endosome function with proliferative signalling and cell-cycle control raises interesting possibilities for these proteins in differentiation and organogenesis.

That Kes1 antagonizes ATF4-dependent transcriptional programmes may have major implications in cancer biology. The mammalian GCN2/ATF4 (homologous with yeast Gcn2/Gcn4) signalling pathway is a highly conserved nutrient, oxidative and hypoxia stress pathway important for tumour survival. Recently, it was demonstrated that activation of the GCN2/ATF4 pathway promotes tumour cell survival and proliferation under amino acid starvation conditions [23]. ATF4 is required to maintain amino acid metabolism in tumour cells [23], and ORPs are implicated in tumour metastasis and cell-cycle progression [34,35]. An association between increased ORP4L (ORP4 long) expression and metastatic cancers is shown. Increased ORP4L mRNA is observed in several cancer cell lines, including cervical-cancer-derived cell lines (HeLa, C33A and SiHa) and a prostate cancer cell line (LNCaP), as well as in breast cancer tissues and lung cancer blood samples [35]. There are 19 human ORPs (encoded by 12 ORP genes)
compared with seven ORPs in yeast, all of which bind sterols [36,37]. We proffer what we believe to be an attractive hypothesis that Kes1-like ORPs antagonize ATF4 activity in mammals, and that enhancing ORP activity in tumours will inhibit their vigour.

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**References**


**Figure 2 | Integration of TGN/endosomal trafficking disruption to transcriptional competence of the GAAC pathway**

Kes1-mediated trafficking arrest leads to manipulation of TGN/endosomal sphingolipid homeostasis which in turn controls cell proliferation, TOR signalling and NH₄⁺ availability. In addition, the sphingolipid signal inhibits transcription initiation/elongation of genes loaded with Gcn4 and RNA polymerase II via action of the CDK8 module of the large SRB Mediator complex. PI-4-P, PtdIns4P.

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