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Inhibition of IκB kinase in Notch signaling activates FOXO3a

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The Notch signaling pathway is highly conserved and plays an important role in the regulation of cellular proliferation, differentiation and apoptosis. Constitutive activation of Notch signaling results in excessive cellular proliferation and a wide range of malignancies. However, Notch can also act as a tumor suppressor, and its inactivation has been associated with an increased risk of spontaneous squamous cell carcinoma.[1] Signaling through the Notch1 receptor is essential for normal T-cell fate specification as well as thymocyte maturation.[2] Approximately 50% of human T-ALLs display activating Notch1 mutations, suggesting an important pathogenetic role for Notch1 in T-ALL.[3] Activating mutations identified in the T-ALL cluster at the heterodimerization domain (HD) and the proline, glutamine, serine and threonine (PEST) NICD1, respectively.

NFκBs are sequestered in the cytoplasm by members of the IκB family (IκB-α, IκB-β and IκB-γ). When the IKK protein kinase complex signalosome phosphorylates IκB, NFκB translocates to the nucleus, dimerizes and engages in the transcription of downstream genes. NFκB-signaling cross-talks with Notch at multiple levels. NFκB signaling results in increased expression of Notch receptors and ligands, leading to augmented Notch signaling,[4] and, conversely, activated Notch signaling upregulates expression of NFκB members.[5] The NFκB pathway is constitutively activated in human T-ALL cells that harbor Notch1 mutations. Moreover, it has been documented that IKK/NFκB signaling is essential for the maintenance of T-ALL, as leukemic cells that are unable to activate the IKK kinase complex rapidly enter apoptosis. Hence, the NFκB pathway is a potential molecular target for the treatment of T-ALL.[6] Accordingly, in the study by Buontempo et al. in this issue of *Cell Cycle*, the authors report the anti-proliferative effects induced by BMS-345541 (a highly selective IKK inhibitor) in three Notch1-mutated T-ALL cell lines and in T-ALL primary cells from pediatric patients. BMS-345541 induced apoptosis and accumulation of cells in the G_2/M phase of the cell cycle via inhibition of IKK/NFκB signaling. Interestingly, they also showed that T-ALL cells treated with BMS-345541 displayed nuclear translocation of FOXO3a and restoration of its functions, including control of p21Cip1 expression levels.

The human FOXO transcription factor family upregulates genes involved in the control of the cell cycle (p27Kip1 and p21Cip1) or in the induction of apoptosis. FOXO3a overexpression inhibits tumor growth in vitro and tumor size in vivo in breast cancer cells. Cytoplasmic location of FOXO3a correlates with poor survival in breast cancer patients.[7] The adverse prognostic value of highly phosphorylated FOXO3a and its cytoplasmic sequestration in acute myelogenous leukemia (AML) have been reported. In tumor cells, AKT, IKK and ERK 1/2 control FOXO3a activation through phosphorylation at different amino acidic residues, thus inducing its translocation from the nucleus to the cytoplasm and its subsequent degradation.

Akt regulates the subcellular localization of FOXO3a by phosphorylation, thereby preventing the protein from translocating to the nucleus and regulating transcription. Constitutive Akt-activation is frequently correlated with cytoplasmatic FOXO3a in breast tumors, and this is associated with decreased patient survival. Nonetheless, FOXO3a is found in the cytoplasm in the absence of activated Akt. Strikingly, IKKβ interacts with and phosphorylates FOXO3a.[8] In the current study, the authors demonstrate that FOXO3a subcellular redistribution is independent of AKT and ERK1/2 signaling. Constitutive phosphorylation of IKK on Ser176/178, which reflects its catalytic activity, is also detectable in the T-ALL models. By using BMS-345541 and also a peptide that directly targets oligomerization of NEMO protein, the authors observed the apoptotic effects and restoration of FOXO3a tumor suppressor functions, regardless of AKT and ERK 1/2 activity. They speculate that in T-ALL the loss of FOXO3a tumor suppressor function could be due to deregulation of IKK, as has been previously demonstrated in other cancer
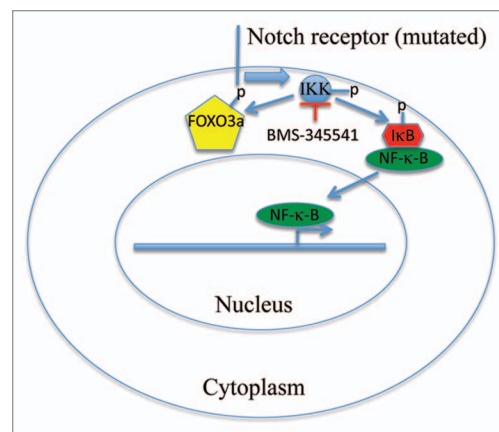

Figure 1. Schematic model depicting the role of IKK inhibition in regulating FOXO3a and NFκB nuclear translocation. In T-ALL, when Notch is mutated and constitutive active, IKK is constant active, which phosphorylates IκB and elicits NFκB nuclear translocation and promote tumorigenesis. The active IKK also sequesters FOXO3a in the cytoplasm. Inhibition of IKK by BMS-345541 triggers FOXO3a nuclear translocation and induces cell cycle arrest and apoptosis.

types. It is well known that, differently from p53, FOXO3a mutations have not yet been found in human tumors, which makes therapeutics activating FOXO3a more appealing than others. For these features, BMS-345541 could be used alone or in combination with traditional therapies in the treatment of T-ALL. Hence, this exciting study suggests that the IKKs might serve as a potential drug target in anticancer therapy, since multiple signal transduction pathways inhibiting proliferation and facilitating cell death could be activated. (**Fig. 1**)

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One stone, two birds: CDK9-directed activation of UBE2A regulates monoubiquitination of both H2B and PCNA

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The modification of proteins by ubiquitin is a highly conserved process, requiring E1 ubiquitin-activating, E2 ubiquitin-conjugating and E3 ubiquitin-ligating enzymes. Protein ubiquitination plays an essential role in regulating key biological processes, including the cellular response to DNA damage.

UBE2A, an E2, is the human ortholog of yeast Rad6 and is essential for the ubiquitination of histone H2B and proliferating cell nuclear antigen (PCNA). Histone H2B monoubiquitination (H2Bub1) is associated with transcriptional elongation on active genes but has also been shown to function in DNA double-strand break (DSB) repair.[1,2] PCNA is ubiquitinated at lysine 164 in response to genotoxic stess, which promotes the recruitment of DNA polymerase η to activate the translesion synthesis DNA repair pathway.[3,4] Both of these processes imply a role for UBE2A in the maintenance of genome integrity.

Cyclin-dependent kinase 9 (CDK9) is a well-characterized component of the positive elongation factor b (P-TEFb) complex, which is involved in transcriptional elongation by phosphorylating the C-terminal domain (CTD) of RNA polymerase II, as well as suppressor of Ty homolog 5 (SUPT5H) and negative elongation factor E (NELF-E). CDK9 also functions in co-transcriptional histone modification,[5,6] mRNA (mRNA) processing, mRNA export and the cellular response to replication stress[7,8] in a manner that is evolutionarily conserved.[9] Loss of CDK9 activity causes an increase in spontaneous levels of DNA damage signaling in replicating cells and a decreased ability to recover from transient replication arrest. CDK9 also localizes to chromatin in response to replication stress, limits the amount of single-stranded DNA in cells under stress and interacts with ATR and other cell cycle checkpoint proteins. However, the mechanism and functional target through which CDK9 functions to maintain genome integrity in response to replication stress has remained unclear. A recent report has identified UBE2A as a novel phosphorylation target for CDK9 and shown that CDK9-dependent activation of UBE2A at conserved serine residue 120 regulates monoubiquitination of both H2B and PCNA,[10] providing mechanistic insight into how CDK9 controls genome integrity.

Pirngruber and Shchebet et al. previously showed that CDK9 activity is essential for maintaining global and gene-associated levels of H2Bub1, at least in part by phosphorylation of serine 2 in the CTD of RNA polymerase II.[6] However, since mutation of serine 2 within the CTD of RNA polymerase II resulted in a milder effect on H2Bub1 than CDK9 knockdown, Shchebet et al. explored whether another CDK9 target might contribute to H2Bub1. Using insight gained from yeast, in which Bur1, the yeast homolog of CDK9, interacts with and phosphorylates Rad6 at serine 120 to promote H2Bub1, they hypothesized that CDK9 may regulate H2Bub1 through UBE2A. Indeed, they found that UBE2A complexes with CDK9 by reciprocal co-immunoprecipitation. In particular, this interaction is specific for CDK9, as another cyclin-dependent kinase, CDK2, does not bind to UBE2A, nor does it regulate H2Bub1. The authors next demonstrated that CDK9 phosphorylates UBE2A at serine 120 both in vitro and in cells. Furthermore, they found that this site-specific phosphorylation by CDK9 regulates UBE2A activity, and CDK9 is required for both UBE2A-dependent H2Bub1 and PCNA monoubiquitination in response to UVC.

The findings in this study provide clarification for how CDK9 regulates H2Bub1. CDK9 phosphorylates RNA polymerase II CTD at serine 2 to recruit the RNF20/40 E3 ubiquitin ligase, which is required for H2Bub1, and phosphorylates UBE2A at serine 120 to increase its activity in regulating H2Bub1. These two pathways are likely not redundant, as abolishing CTD serine 2 phosphorylation leads to an incomplete loss of H2Bub1.[6] However, it will be of interest to further clarify the individual contribution of these two pathways and their potential crosstalk in the maintenance of H2Bub1. This study also provides the first link between CDK9 and PCNA monoubiquitination. The dual function of CDK9-UBE2A signaling in modulating both H2Bub1 and PCNA monoubiquitination provides further evidence for a role for CDK9 in genome maintenance. From a clinical perspective, this study offers a mechanistic rationale for the application of CDK9 inhibitors, such as flavopiridol,

in cancer therapy. Moreover, by understanding how CDK9 functions in controlling H2B and/or PCNA monoubiquitination to maintain genome integrity, insights may be gained into targeting these proteins or their interactions to enhance the efficacy of more traditional cancer therapies.

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Osh6 links yeast vacuolar functions to lifespan extension and TOR

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Almost all organisms age–the aging process is both genetically determined and can be modified by the environment. Lifespan extension by dietary restriction (DR) is observed in evolutionarily distant species from yeast to mammals. Not only are the phenomena of aging and DR conserved, but at least some mechanisms and genes are evolutionarily conserved, which may pave the way to manipulate human aging.[1] For example, TOR (target of rapamycin) mediates aging and, when suppressed, triggers anti-aging processes in many species. Moreover, identifying genes that modulate the potential for cell division is of great interest, given that changes in the number of times that cells divide have been associated with longevity manipulations in mammals (including DR).[2]

Sterols are hydrophobic molecules present in all cellular organisms. For instance, cholesterol is an essential structural component of cellular membranes of mammals and several of its derivates have additional hormonal and signaling functions. Oxysterols are oxygenated derivates of cholesterol. Oxysterol-binding protein (OSBP)-related protein (ORP) family members are present in numerous copies from yeast to man, suggesting that this protein family has fundamental functions in eukaryotes. OSBP and ORPs regulate lipid metabolism, vesicle transport and various signaling pathways[3] and may specifically mediate lipid exchange at membrane contact sites.

The lifespan-extending effect of DR has often been shown to be mediated by specific genes and to be accompanied by discrete changes in gene expression as well as metabolic reprogramming. Both lipid metabolism and cellular recycling activities have been demonstrated to be essential for lifespan extension in numerous species. For example, DR suppresses sterol synthesis from yeast to mammals,[4] while it induces some form of autophagy, a mighty housekeeping mechanism utilizing lysosomes within its power to recycle various kinds of molecules and cellular structures. Vacuoles, the yeast equivalent of mammalian lysosomes, are highly dynamic organelles that fuse and divide in response to environmental or intrinsic cues. Mutants with defects in vacuolar fusion (such as ypt7Δ, nyv1Δ, vac8Δ, or erg6Δ) are either short-lived or do not appear to respond to DR.[5]

While mammals have 12 OSBPs, the yeast genome encodes seven oxysterol-binding protein sequence homologs (OSH). Deletion of any OSH gene alone does not impact on vacuolar morphology, yet deletion of all results in highly fragmented vacuoles, a sign of defective vacuole fusion. Gebre et al. now show that overexpression of OSH family member OSH6 in yeast can complement the vacuole fusion defect of nyv1Δ but not erg6Δ or vac8Δ. Thus, Osh6 mediates vacuolar fusion, which depends on ergosterol (Erg6), and the protein anchor Vac8. In contrast, overexpression of another OSH-family member, OSH5, exacerbated fragmentation and decreased lifespan in wild-type cells. It is interesting to note that OSH5 expression progressively increases with age, and Osh6 overexpression blocked this age-dependent change in OSH5 levels. Also, elevated Osh6 maintains the enrichment of Vac8 in microdomains of vacuolar membranes with advancing age, which is required for vacuole fusion. Intriguingly, exactly at the age when the longevity protein Sir2 declines, Osh6 protein levels also decline.[6]

Furthermore, Gebre et al. showed that PERG6-OSH6 (ERG6 promoter driving OSH6 overexpression) dramatically extends the lifespan of wild-type and nyv1Δ mutants. tor1Δ mutants are also long-lived, though not so long as PERG6-OSH6. Surprisingly, PERG6-OSH6 tor1Δ double mutant had a very short lifespan. PERG6-OSH6 mutants were more sensitive to TOR inhibitors, indicating that TOR is less active in this strain.[6] OSH6 overexpression downregulates total cellular sterol levels, just like DR. Osh6 binds PI3P and PI(3,5) P2 which are vacuole-specific lipids.[7] As such, Osh6 might promote vacuole fusion by regulating the transports and/or distribution of sterols to the vacuolar membranes. But where are the sterols coming from? Numerous overexpression mutants with effects in vacuolar morphology are involved in endocytosis.[8] Similarly, Osh6's coiled-coil domain interacts with Vps4, which is located in endosomes. TOR complex 1 (TORC1) also sits on endosomes as well as on vacuoles and actively catalyzes vacuolar scission.[9] Osh6 may therefore (1) transport sterols from late endosomes to the vacuolar membrane (**Fig. 1**), which increases the homotypic fusion ability of vacuoles, and (2) averaging the lipids between late endosome and vacuoles promotes also late-endosome-to-vacuole fusion.
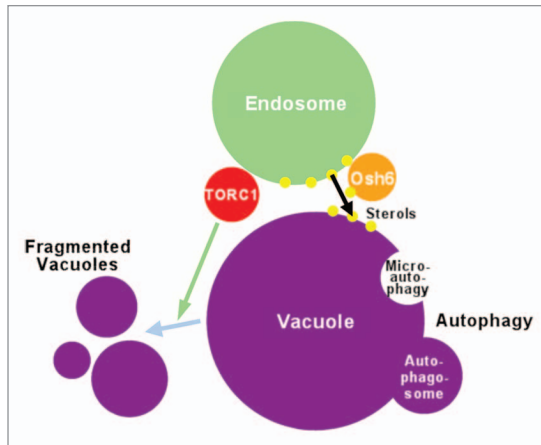
Figure 1. Putative mechanism of the lifespan extension conferred by Osh6 overexpression. TORC1 promotes vacuolar scission and therefore fragments vacuoles. In contrast, Osh6 enhances vacuolar fusion and might be doing this by transporting sterols from the endosomes to the vacuolar membrane. Improved vacuolar morphology then promotes autophagy. Thus, Osh6 appears to counteract TORC1 activity.

Overall, Gebre and colleagues link the vacuole to lifespan extension, perhaps via TOR, and reveal that vacuole fusion is both necessary and sufficient for lifespan extension.

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Creative destruction of the microtubule array

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Typical cells contain a dense array of microtubules that serves as a structural backbone and also provides a substrate against which molecular motor proteins generate force. Cells transitioning through the cell cycle or undergoing significant morphological changes must be able to tear apart the microtubule array and reconstruct it into new configurations, either partially or completely. The microtubule field was revolutionized in the 1980s with the introduction of the dynamic instability model,[1] now broadly recognized as a fundamental mechanism by which microtubule populations are reconfigured.[2] Dynamic instability involves the catastrophic disassembly of microtubules, generally from their plus ends, as well as the rapid reassembly of microtubules and selective stabilization of particular ones. Microtubules can be stabilized along their length by binding to various proteins and can be attached at their minus ends to structures such as the centrosome and "captured" at

their plus ends by proteins in the cell's cortex.[2] Given the contribution of these stabilizing and anchoring factors, additional mechanisms beyond dynamic instability are required to tear down previous microtubule structures so that new ones can be constructed. Borrowing from the field of economics, we refer to this as creative destruction.

Various proteins such as stathmin[3] and kinesin-13[4] contribute to creative destruction by promoting loss of tubulin subunits from the ends of the microtubules. We find especially interesting a category of AAA enzymes called microtubule-severing proteins that use the energy of ATP hydrolysis to yank at tubulin subunits within the microtubule, thereby causing the lattice to break.[5] If this occurs along the length of the microtubule, the microtubule will be severed into pieces. If this occurs at either of the two ends of the microtubule, the microtubule will lose subunits from that end. The first discovered and best-studied

microtubule-severing proteins are katanin and spastin.

Thanks to David Sharp and his colleagues at Albert Einstein College of Medicine, as well as other workers in the field, we now know that cells express at least five other AAA proteins with potential microtubule-severing properties, on the basis of sequence similarity to katanin and spastin in the AAA region.[5] Two of these, called katanin-like-1 and katanin-like-2, are very similar to katanin. The three others are similar to one another, collectively termed fidgetins (fidgetin, fidgetin-like-1 and fidgetin-like-2). One possibility is that all seven of the microtubule-severing proteins are regulated similarly and are functionally redundant with one another. A more compelling possibility is that, while there is some functional redundancy, there is also a division of labor, with each severing protein displaying distinct properties and carrying out its own duties. Thus far, Sharp's studies on mitosis support the

latter scenario, with katanin, fidgetin and spastin having characteristic distributions within the spindle, resulting in unique phenotypes when depleted.[6]

In a new article, Sharp's group has confirmed that fidgetin has microtubule-severing properties. Interestingly, fidgetin depolymerizes microtubules preferentially from the minus end.[7] In addition, the new work shows that in human U2OS cells, fidgetin targets to the centrosome, where most minus ends of microtubules are clustered, suggesting a scenario by which fidgetin suppresses microtubule growth from the centrosome as well as attachment to it. Consistent with this scenario, the authors show that experimental depletion of fidgetin reduces that speed of poleward tubulin flux as well as the speed of anaphase A chromatid-to-pole motion and also results in an increase in both the number and length

of astral microtubules. Notably, this contrasts with katanin, which favors the plus ends of microtubules, for example, at the chromosome during cell division[6] and at the leading edge of motile cells.[8]

The authors close their article by pointing out that microtubule-severing is important beyond mitosis, for example, in the restructuring of the microtubule array in neurons and migrating cells, and we would point to plants as well.[9] We previously described a mechanism called "cut and run," wherein the severing of microtubules is important for motility within the microtubule array, as short microtubules are more mobile than long ones.[9] Now, inspired by the work of Sharp and colleagues, we envision "creative destruction" as another way of understanding the crucial roles played by a diversity of microtubule-severing proteins in cells.

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