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Cell Cycle News & Views

Length matters: C-terminal tails regulate Mdm2–MdmX complexes

Comment on: Dolezelova P, et al. Cell Cycle 2012; 11:953–62; PMID:22333590; http://dx.doi.org/10.4161/cc.11.5.19445

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Mechanisms controlling the p53 regulatory network remain the focus of numerous investigations in hopes of identifying more robust cancer therapies. Both Mdm2 and MdmX are found overexpressed in tumors with wild-type p53 and represent a key molecular device modulating p53 function. Thus, examining the interplay between these three proteins becomes highly relevant in the search for new pharmacological interventions in oncology.

Mdm2 is a RING-type E3 ubiquitin ligase capable of forming homo-oligomers and hetero-oligomerization with MdmX via the extreme C termini of their RING domains. Since its discovery 15 years ago, MdmX has been assigned many roles in the regulation of p53, either on its own or in concert with Mdm2. While clearly an essential negative regulator or p53 in development, its lack of intrinsic ubiquitin ligase activity has made the mechanism of p53 regulation more elusive than in the case of Mdm2. The capacity of MdmX to stimulate Mdm2-mediated p53 ubiquitination was first reported in 2003.[1] Subsequent biochemical comparisons of the activity of Mdm2–MdmX complexes showed that not only does the presence of MdmX in the complex alter the substrate specificity of the holo-enzyme, it also allows for poly-ubiquitin chain formation on p53 (modification required for nuclear exclusion and degradation of p53).[2-4]

In vitro observations describing the importance of the MdmX RING domain in regulation of p53 turnover have now gained in vivo experimental support from the two knock-in animal models.[5,6] Consistent with the notion that MdmX is an essential component of p53 polyubiquitination/proteasomal degradation pathway, mice expressing either a point mutant in the MdmX RING domain or a RING domain deletion mutant succumbed to a p53-dependent embryonic lethality. These data implicate the RING domain of MdmX as the sole region of importance in the ability of MdmX to regulate p53 and, by extension, the Mdm2-MdmX complex (and not the Mdm2 homodimer), as the principle negative regulator of p53 activity during development.

The growing body of evidence describing the presence of MdmX in the complex as crucial for target selectivity as well as the processivity of the holoezyme somewhat flies in the face of the existing structural data. Two published structures of the Mdm2 homodimer and Mdm2/MdmX heterodimer indicate virtually no difference in the complexes.[7,8] In the absence of structural differences, how then are such significant differences in function accomplished?

A hypothesis unifying structural and functional data is brought forth by a very intriguing study from the Uldrijan group, which systematically looks at the differences between complex formation and activity of Mdm2 and MdmX.[9] Phylogenetic analysis showed that the last cystein of the RING domain is followed by exactly 13 amino acids in all Mdm orthologs of vertebrate origin. Based on this, the authors hypothesized that not only the sequence of the C-terminal tails, but also their exact length are of central importance to the function of the complexes. Subsequent investigation of the ability of Mdm2 and MdmX proteins, which have been extended at the C terminus by 5, 14 or 18 amino acids, was designed to test the importance of the length of the C-terminal extensions. To the researchers surprise, when examined based on their ability to hetero-oligomerize and ubiquitinate p53, Mdm2 proteins behaved differently depending on whether the oligomeric partner was Mdm2 or MdmX.

Dolezelova et al. present unexpected experimental evidence for the heterocomplex being structurally and functionally distinct from the Mdm2 homodimer, while providing a mechanism for the observed in vivo functional differences between the complexes. Although the work casts slight doubt on the complete accuracy of the existing structures, it nicely aligns with the above-mentioned results, showing the singular importance of the MdmX RING domain in the activity of the holoenzyme. In light of these results, additional structural studies that will take in to account reported differences between the complexes will undoubtedly be informative and contribute to our understanding of the biochemistry of RING-type ubiquitin ligases and the mechanisms regulating p53 in cells.

References

1. Linares LK, et al. Proc Natl Acad Sci USA 2003; 100:12009-14; PMID:14507994; http://dx.doi.org/10.1073/pnas.2030930100.
2. Okamoto K, et al. FEBS Lett 2009; 583:2710-4; PMID:19619542; http://dx.doi.org/10.1016/j.febslet.2009.07.021.
3. Kawai H, et al. Cancer Res 2007; 67:6026-30; PMID:17616658; http://dx.doi.org/10.1158/0008-5472.CAN-07-1313.
4. Wang X, et al. J Biol Chem 2011; 286:23725-34; PMID:21572037; http://dx.doi.org/10.1074/jbc.M110.213868.
5. Huang L, et al. Proc Natl Acad Sci USA 2011; 108:12001-6; PMID:21730163; http://dx.doi.org/10.103/pnas.1102309108.
6. Pant V, et al. Proc Natl Acad Sci USA 2011; 108:11995-2000; PMID:21730132; http://dx.doi.org/10.1073/pnas.1102241108.
7. Kostic M, et al. J Mol Biol 2006; 363:433-50; PMID:16965791; http://dx.doi.org/10.1016/j.jmb.2006.08.027.
8. Linke K, et al. Cell Death Differ 2008; 15:841-8; PMID:18219319; http://dx.doi.org/10.1038/sj.cdd.4402309.
9. Dolezelova P, et al. Cell Cycle 2012; 11:953-62. PMID:22333590; http://dx.doi.org/10.4161/cc.11.5.19445.

pRb or its cousins: Who controls the family business?

Comment on: Bazarov AV, et al. Cell Cycle 2012; 11:1008–13; PMID:22333593; http://dx.doi.org/10.4161/cc.11.5.19492

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More than 90% of human cancers are of epithelial origin. Cellular senescence of human mammary epithelial cells (HMECs) is an important barrier that protects cells from immortalization; the first step in breast cancer development.[1] Although induction of tumor suppressor p16 is not evident in some types of normal human fibroblasts undergoing senescence,[2] in cultured HMECs, senescence occurs by a robust p16 induction, and cells that acquire silencing of *p16Ink4a* locus eventually proliferate and undergo senescence again by telomere shortening in a p53-dependent manner.[1] Therefore, p16 induction is a critical barrier to immortalize HMECs in culture. p16 inhibits kinase activity of Cdk4/6-cyclinD complexes, which inactivate three pRb family proteins: pRb, p107 and p130. However, the relative contribution of these three pRb family proteins to HMEC senescence is not well understood.

In a recent issue of *Cell Cycle*, Bazarov et al. examined the role of each pRb family protein in p16-mediated senescence in breast cancer cell lines and in HMECs (**Fig. 1**).[3] They showed that knockdown of each of the three pRb family proteins individually did not abrogate senescence mediated by ectopically expressed p16 in the breast cancer cell lines MDA-MB-231 and MCF7. However, the senescence induced by ectopic p16 was abrogated if they introduced E7, which inactivates all three pRb family proteins. Their data suggest that two of pRb family proteins can compensate for the loss of each pRb family protein to induce p16-mediated senescence in these cancer cells. The remaining question is whether all three pRb family members play an additive role, and whether the inactivation of at least two members of the pRb family is required to overcome p16-induced senescence in breast cancer cells. On the other hand, they showed that abrogation of pRb, but not of p107 and/or p130, attenuates senescence in HMECs, suggesting a non-redundant critical role of pRb in HMEC senescence. These data are consistent with a recent report demonstrating that pRb has a non-redundant role in repressing DNA replication during H-ras-induced senescence of human fibroblasts,[4] and explain why pRb, but not p107 or p130, is frequently mutated in cancer. Interestingly, although abrogation of pRb is critical for HMECs escaping senescence, simultaneous depletion of pRb together with either p107, p130 or both accelerates bypass of senescence. This suggests that p107 and p130 help pRb to trigger/maintain HMEC senescence in culture and possibly in vivo. Although each pRb family protein preferentially binds to different members of the E2F family,[5] the contribution of each E2F family protein in escaping p16-mediated senescence remains unclear. Therefore, it will be interesting to see whether the critical role of pRb, and a supportive role of p130 and p107, in p16-mediated HMEC senescence depend on how each pRb family protein interacts with an E2F family protein.

Bazarov et al. also showed that even aggressive p53-negative breast cancer cells undergo cellular senescence upon ectopic p16 expression. These results are quite encouraging from an epigenetic therapy point of view. Silencing of p16 often occurs in breast cancer cells via promoter methylation. During DNA replication, cells require new p16 promoter methylation to keep p16 silenced. The observations of Bazarov et al. suggest that we may be able to stop the growth of even aggressive p53-negative breast cancers in patients by inducing p16 expression in cancer cells using DNA methylation inhibitors. Back to the question of running family business: "it appears that pRb is still the boss, but in some cases, it may get a helping hand from his cousins- p107 and p130."

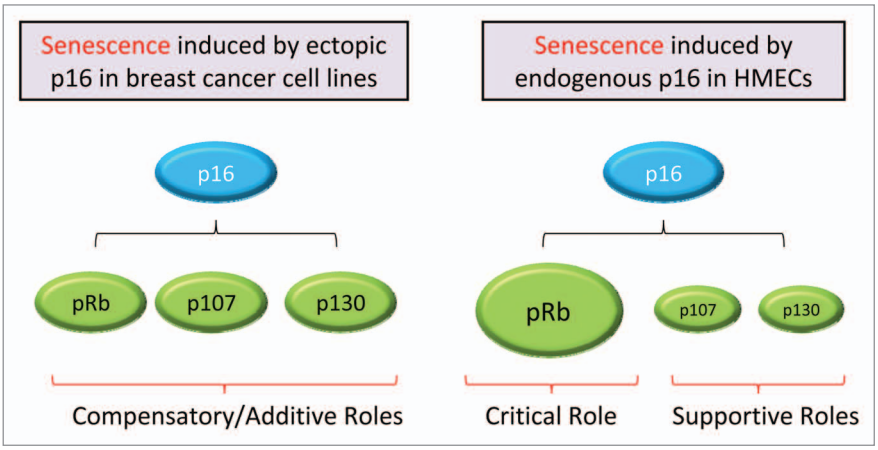

Figure 1. Contribution of pRb family proteins to p16-mediated senescence in breast cancer cells and HMECs. Knockdown of each of the three pRb family proteins in breast cancer cells does not abrogate ectopic p16-induced senescence, suggesting that either two of pRb family proteins can compensate for the loss of each pRb family proteins or all three of pRb family proteins play an additive role in p16-mediated senescence in breast cancer cells. On the other hand, knockdown of pRb, but not of p107 or p130, abrogates HMEC senescence, suggesting a non-redundant critical role for pRb in senescence of HMECs. However, the knockdown of either p107 or p130, in conjunction with pRb depletion, abrogates HMEC senescence more efficiently than pRb knockdown alone. This suggests a supporting role for p107 and p130 in maintaining HMEC senescence.

References

1. Stampfer MR, et al. Cancer Lett 2003; 194:199-208; PMID:12757978; http://dx.doi.org/10.1016/S0304-3835(02)00707-3.
2. Itahana K, et al. Mol Cell Biol 2003; 23:389-401; PMID:12482990; http://dx.doi.org/10.1128/MCB.23.1.389-401.2003.
3. Bazarov AV, et al. Cell Cycle 2012; 11:1008-13; PMID:22333593; http://dx.doi.org/10.4161/cc.11.5.19492.
4. Chicas A, et al. Cancer Cell 2010; 17:376-87; PMID:20385362; http://dx.doi.org/10.1016/j.ccr.2010.01.023.
5. Classon M, et al. Nat Rev Cancer 2002; 2:910-7; PMID:12459729; http://dx.doi.org/10.1038/nrc950.

Milking the stroma in triple-negative breast cancer

Comment on: Witkiewicz AK, et al. Cell Cycle 2012; 11: 1108–1117; PMID:22313602; http://dx.doi.org/10.4161/cc.11.6.19530

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Investment in the post-genomic molecular dissection of breast cancer has resulted in an emphasis on prognostic and predictive markers, signatures derived to stratify the disease and the drive to generate targeted therapies. However, there remain significant challenges to individualize therapeutic targeting and improve the prognosis for the thousands of women who die each year from the heterogeneous range of breast cancers. This is particularly true for poor prognosis "triple-negative" breast cancers (TNBC), most prevalent in young and African American women, lacking the established therapeutic targets of estrogen receptor, progesterone receptor or HER2.

Research has largely focused on the epithelial component of breast cancer rather than the tumor microenvironment, now recognized as a key hallmark of cancer.[1] In vitro, animal models and observations on clinical material[2] are now moving to consider physiological mechanisms by which stromal cells may influence breast epithelial and carcinoma cells.

Witkiewicz et al.[3] build on published evidence from the Lisanti group that cancer cells secrete hydrogen peroxide, initiating oxidative stress and aerobic glycolysis in tumor stroma, with L-lactate secretion from cancer-associated fibroblasts fueling oxidative mitochondrial metabolism in epithelial cancer cells: the "reverse Warburg effect."

They demonstrate stromal monocarboxylate transporter 4 (MCT4), detected by immunohistochemistry, as a functional marker of stromal hypoxia, oxidative stress, aerobic glycolysis and L-lactate efflux. High stromal MCT4 expression (but, critically, not epithelial MCT4) was associated with poor prognosis in TNBC patients. Combined high stromal MCT4 and loss of stromal caveolin-1 identify particularly poor prognostic TNBC.

Thus, development of cancer may not lie solely in genetic or epigenetic epithelial changes, but with acquired functional changes in the stromal infrastructure of the breast. This supports the concept of epithelial malignant changes consequent with ecological and evolutionary opportunity.[4]

The "parasitic" character of tumor cells feeding off stromal cells highlights the need to seriously consider both ecological and biophysical concepts.[5] We need to think beyond "intraspecific" competition among clonal subpopulations in the tumor and to consider tumor and stromal cells as distinct populations in a cancer ecosystem, with a range of "interspecific" competitive, exploitative and opportunistic interactions. Furthermore, the reverse Warburg effect relies on the inefficient diffusion of nutrients from stromal cells to tumor cells in a complex three-dimensional space. The extracellular space is brought to the foreground, and physical properties of molecular transport in this space may have as much impact on tumor growth as intricate cellular processes.

The importance of the spatial arena is also apparent when contrasting the reverse Warburg effect with angiogenesis. In the former, tumor cells are exploiting their local environment, which will presumably be of limited yield, whereas angiogenesis taps the nutrients of the entire organism—an effectively infinite reservoir for a growing tumor. In the reverse Warburg effect, a balance of ecological and biophysical factors underpins the sustainability of this mode of cancer nutrition.

A two-compartment model coupling oxidative epithelial cells with glycolytic fibroblasts reflects increased expression of hypoxia-associated genes as a component part of prognostic stromal signatures.[6] Further evidence of stromal/epithelial interaction comes from evidence that the effects of radiation on normal breast epithelium in vivo is at least partially dependent on the stromal context.[7]

Manipulation of the tumor microenvironment to promote an anticancer phenotype challenges the cancer treatment paradigm. The long-established antidiabetes biguanide drugs offer a low-toxicity opportunity to disrupt the reverse Warburg effect. Metformin may target the cancer mitochondria[3] and phenformin induce stromal sclerosis, at least in a breast cancer xenograft model,[8] in addition to in vivo AMPK pathway and insulin-mediated systemic effects of metformin in women with breast cancer.[9]

The reverse Warburg effect challenges our therapeutic focus on breast cancer epithelium. Stromal MCT4 expression with caveolin-1 loss identifies poor prognostic TNBC patients and emphasizes the roles of the tumor microenvironment and ecological interactions between distinct populations of cells. The challenges now revolve around therapeutic manipulation of the stroma/epithelial interaction and the extracellular space, and testing these concepts in pre-invasive and metastatic settings where stromal changes may provide tissue niches of evolutionary opportunity for malignant cells.

References

1. Hanrahan D, et al. Cell 2011; 144:5646-74
2. Place AE, et al. Breast Cancer Res 2011; 13:227; http//breast-cancer-research.com/content/13/6/227; PMID:22078026; http://dx.doi.org/10.1186/bcr2912.
3. Witkiewicz AK, et al. Cell Cycle 2012; 11:1108-17; PMID:22313602; http://dx.doi.org/10.4161/cc.11.6.19530.
4. Gatenby RA, et al. Nat Rev Cancer 2011; 11:237-8; PMID:21548400; http://dx.doi.org/10.1038/nrc3036.
5. Moore NM, et al. Phys Biol 2011; 8:010302; PMID:21368346; http://dx.doi.org/10.1088/1478-3975/8/1/010302.
6. Finak G, et al. Nat Med 2008; 14:518-27; PMID:18438415; http://dx.doi.org/10.1038/nm1764
7. Coates PJ, et al. Cancer Res 2010; 70:1-8; http://dx.doi.org/10.1158/0008-5472.CAN-10-1118.
8. Appleyard MVCL, et al. Br J Cancer 2012; 106:1117-22; PMID:22361631; http://dx.doi.org/10.1038/bjc.2012.56.
9. Hadad S, et al. Breast Cancer Res Treatment 2011; 128:783-94.

Stress, specificity and the NEDD8 proteome

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In an exciting and surprising paper in a recent issue of *Cell Cycle,* Leidecker et al. show that the balance between protein modification by ubiquitin or the ubiquitin like protein NEDD8 is dramatically altered by cellular stress. In a variety of conditions that reduce the concentration of free ubiquitin, a very dramatic increase in protein modification by neddylation is revealed. Importantly, this process is shown to arise as NEDD8 is activated under these conditions by the ubiquitin-activating enzyme Ube1 and not by the typical NEDD8 specific EI enzyme, NAE. This results in many proteins in stressed cells being modified by mixed ubiquitin NEDD8 chains, which is highly relevant in the development of novel cancer therapeutics, as the NAE specific inhibitor MLN4924[2]does not block this new pathway despite its promising anticancer activity.

Initial comparative studies on the ubiquitin and ubiquitin-like (Ubl) protein pathways have established that each pathway has separate and specific enzymes both for activating the Ubl and for removing it.[3] In the case of NEDD8, the E1 is NAE; the E2s are Ubc12 and Ube2F, and the E3s include the Rbx1 and Rbx2 RING finger proteins as well as members of the DCN family of proteins. The first studies of the NEDD8 system suggested that there were very few substrates for this modification, with most emphasis placed on the cullin proteins. The cullins are components of the cullin-RING ligases (CRLs) that are responsible for the ubiquitylation of many critical substrates, for example, oncoproteins such as cyclin E and c-myc. The cullins are modified by neddylation, which increases the E3 activity of the CRLs, probably through structural alterations that free the Ring domain of the E3 and/or by blocking the binding of inhibitory proteins such as CAND 1.[4,5] Recently, many new substrates and E3 ligases for NEDD8 have been uncovered, with initial studies identifying p53 and Mdm2 as substrates for neddylation, and Mdm2 as a E3 ligase for both NEDD8 and ubiquitin.[6] Proteomic approaches have now identified many more substrates, notable among them being the ribosomal proteins involved in signaling to p53.[7,8] In the current study, the authors found that a high level of NEDD8-conjugated proteins were rapidly induced by proteasome inhibition with MG132, but that this reaction was not inhibited by MLN4924, even while the same compound was blocking cullin neddylation. This meant that another E1 had to be in play for the neddylation of these new substrates, and knockdown of Ube1 (which was known to be able to activate NEDD8 in vitro)[9] showed that it was, indeed, responsible. Exploring further stress signals showed that this increased neddylation response was induced by heat shock and by elevated levels of reactive oxygen species (ROS). Since all of these stress pathways reduce free ubiquitin levels, the authors asked if NAE-independent neddylation could be triggered simply by reducing free ubiquitin levels. The clearly positive results of this study suggested that competition with ubiquitin for Ube1 may normally limit Ube1 activation of NEDD8 and the neddylation of non-cullin substrates (**Fig. 1**).

In stress conditions then, when free ubiquitin levels fall, Ube1 acts as a sensor of this state and neddylation increases. Why would this be useful? The speculation is that the modification of substrate proteins by NEDD8 may help the cell to cope with stress signals, for example, by promoting cell survival through inhibition of the degradation of very labile pro-survival proteins, such as Mcl-1. After the stress signal abates, the many effective de-ubiquitinating and de-neddylating enzymes can come into play to restore homeostasis. Improved mass spectrometry methods developed in this paper using Lys-C to digest neddylated proteins allow one to distinguish NEDD8 modification from ubiquitination. This helps to further refine our knowledge of this fascinating system, but, meanwhile, protein neddylation may provide a new biomarker for cellular stress. Many critical issues remain to be resolved: are there proteins with ubiquitin/NEDD8 binding domains that specifically recognize the ubiquitin NEDD8 hybrid chains that result from these stress signals? Which E2s and E3s are responsible for stress-induced neddylation? Should Ube1 inhibitors be developed to complement the NAE inhibitor in cancer treatments, or would they prove too toxic? The next few years promise to reveal critical insights into the crosstalk between the different Ubl pathways.

References

1. Leidecker O, et al. Cell Cycle 2012; 11:1142-50; PMID:22370482; http://dx.doi.org/10.4161/cc.11.6.19559.
2. Soucy TA, et al. Nature 2009; 458:732-6; PMID:19360080; http://dx.doi.org/10.1038/nature07884.
3. Kerscher O, et al. Annu Rev Cell Dev Biol 2006; 22:159-80; PMID:16753028; http://dx.doi.org/10.1146/annurev.cellbio.22.010605.093503.
4. Watson IR, et al. Cancer Cell 2011; 19:168-76; PMID:21316600; http://dx.doi.org/10.1016/j.ccr.2011.01.002.
5. Duda DM, et al. Curr Opin Struct Biol 2011; 21:257-64; PMID:21288713; http://dx.doi.org/10.1016/j.sbi.2011.01.003.
6. Xirodimas DP, et al. Cell 2004; 118:83-97; PMID:15242646; http://dx.doi.org/10.1016/j.cell.2004.06.016.
7. Xirodimas DP, et al. EMBO Rep 2008; 9:280-6; PMID:18274552; http://dx.doi.org/10.1038/embor.2008.10.
8. Sundqvist A, et al. EMBO Rep 2009; 10:1132-9; PMID:19713960; http://dx.doi.org/10.1038/embor.2009.178.
9. Whitby FG, et al. .J Biol Chem 1998; 273:34983-91; PMID:9857030; http://dx.doi.org/10.1074/jbc.273.52.34983.

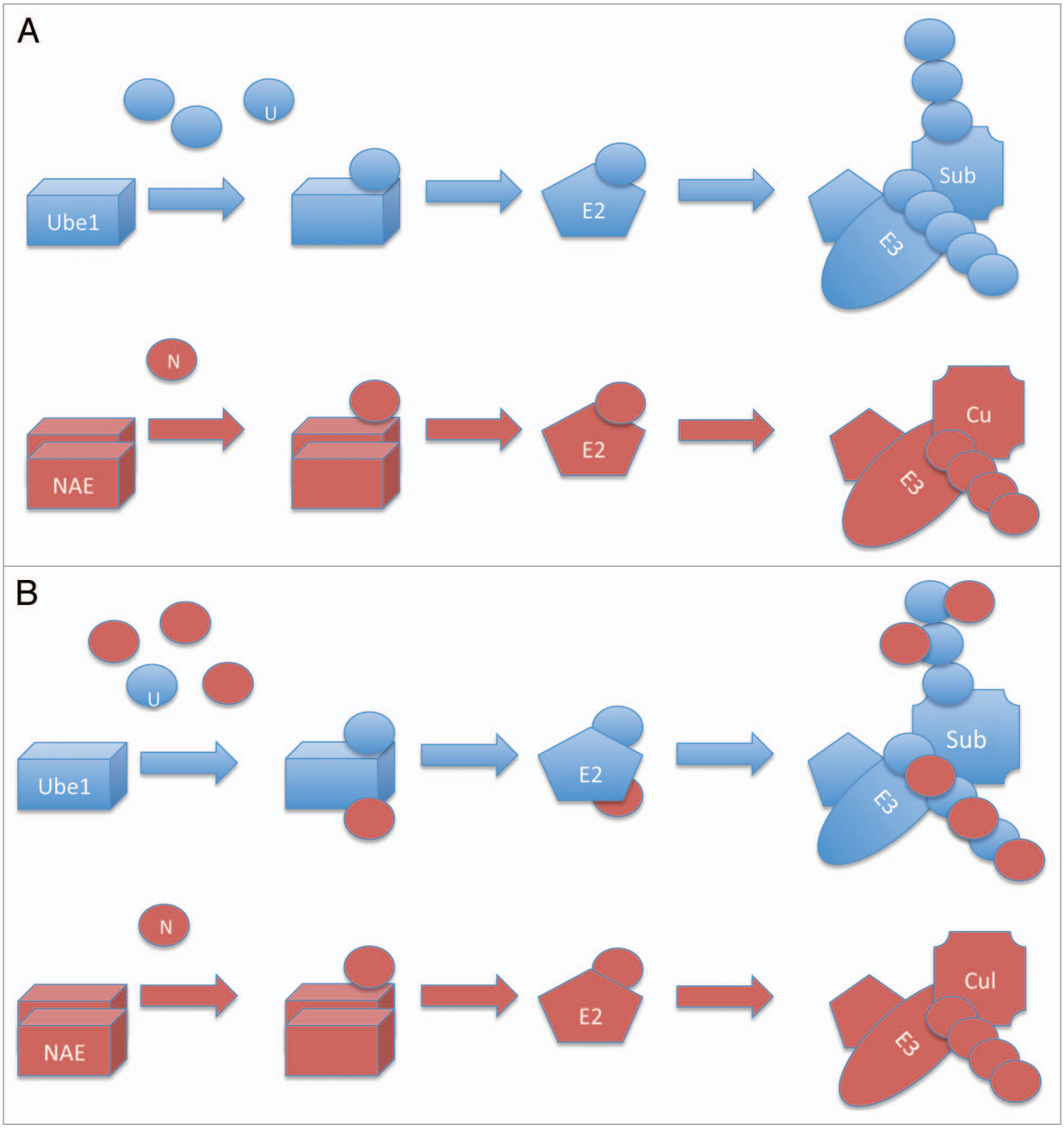

Figure 1. Nedd8 pathway and stress. (A) In unstressed cells, two parallel and non-overlapping pathways are in play. Nedd8 activation is through the action of NAE, while ubiquitin is activated by Ube1. Substrate selectivity of the E2 and E3 results in many proteins being ubiquitinated, but few are Nedd8-modified, notably, the cullins. (B) Low free ubiquitin levels in stress conditions results in Nedd8 being activated by the ubiquitin Ube1 as well as NAE1. This, in turn, results in a large increase in the variety of protein substrates that are NEDD8-modified, in addition to the cullins.