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Review

The emerging role of Mule and ARF in the regulation of base excision repair

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ABSTRACT

The ARF (Alternative Reading Frame) protein is encoded in the Ink4a locus of human chromosome 9 that is frequently mutated in cancer cells. It was recently demonstrated that ARF is induced in response to DNA damage and inhibits, by direct interaction, the E3 ubiquitin ligase Mule that regulates p53 protein levels. Mule inhibition leads to p53 accumulation and activates cellular DNA damage responses. Mule has also recently been identified as a major E3 ubiquitin ligase involved in the regulation of DNA base excision repair. In this review, we will summarise the major properties of Mule and ARF and their roles in the coordination of DNA repair and DNA replication.

1. Mule structure

In mammalian cells, cellular protein levels are regulated through the ubiquitin–proteasome pathway. Proteins targeted for degradation are marked with a chain of ubiquitin molecules, achieved through a cascade of ubiquitin activating enzymes, which are attached to a specific lysine residue(s) in the protein. Firstly, ubiquitin (Ub) is activated through an ATP-dependent reaction by a ubiquitin activating enzyme (E1) to form the E1-Ub thioester. Secondly, the activated ubiquitin is delivered to an ubiquitin conjugating enzyme (E2) and finally, a complex is formed between the E2-Ub thioester, the target protein and a ubiquitin ligase (E3) that conjugates ubiquitin to the protein. Polyubiquitylated proteins are recognised by the 26S proteasome that unfolds the protein, removes the polyubiquitin chains and degrades the protein. It is thought that several hundred human E3s exist that target specific proteins for ubiquitylation, although there are three major classes of E3s: RING-finger, HECT domain and U-box E3s that serve to bring together the conjugated E2 and the substrate protein (for review see [1]). The subject of this review, the E3 ubiquitin ligase Mule (also known as ARF-BP1, E3Histone, UREB1, HUWE1, HectH9, and LASU1) which is a 482-kDa protein consisting of 4374 amino acids, belongs to the family of HECT (*h*omologous to *E*6-AP *c*arboxyl *t*erminus) domain E3 ubiquitin ligases [2]. The members of this family are defined by the presence of the conserved C-terminal HECT domain which consists of approximately 350 amino acids and is responsible for the E3s catalytic activity (Fig. 1). This domain has been shown to contain an essential conserved cysteine residue that forms the thioester complex with the E2-activated ubiquitin molecule, prior to the ubiquitin transfer to the lysine residue on a substrate [3,4]. Later studies have established that the HECT domain might be responsible for both the topology of the ubiquitin chain formation as well as the mechanism of its assembly [5], whereas the substrate specificity of HECT E3 ligases is provided by their N-terminal sequences outside the HECT domain [6,7]. A recent structural study [8] has shown that the HECT domain of Mule (amino acid residues 3993–4374) is composed of two sub-domains. Both sub-domains are connected by a flexible linker that provides conformational flexibility essential for ubiquitin chain transfer. One of them contains the catalytic cysteine residue (Cys4341) and the other sub-domain includes the E2 binding region (Fig. 1). Additionally, a single α-helix (residues 3993–4012) was identified within the E2 binding region of Mule, deletion of which resulted in drastic reduction of the HECT domain stability, and surprisingly in a significant increase in self-ubiquitylation. This effect has been explained by increased conformational flexibility of the mutant lacking the α-helix which may enhance the efficiency of certain catalytic steps, such as the E2 enzyme – HECT interaction of product release by shifting the conformational state of the protein into a more favorable orientation [8].

Mule has several protein–protein interaction domains indicating its involvement in multiple cellular transactions. Mule has a ubiquitin-associated domain (named UBA, residues 1317–1355), a short sequence motif containing a hydrophobic surface patch

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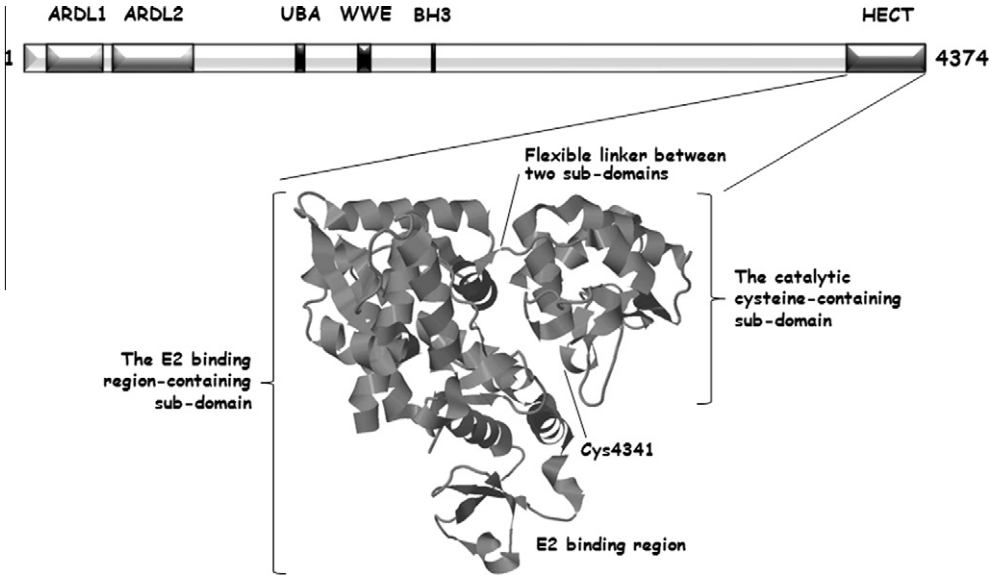

Fig. 1. Structure of the Mule protein. Schematic representation of the Mule protein indicating several domains and its 3D-structure (PDB code 3H1D) showing a few important structural elements required for protein activity and its stability (as described in [8]).

which has been proposed to interact with that of the ubiquitin molecule [9]. The Mule protein also contains a WWE domain (residues 1617–1678) which was predicted to be involved in protein–protein interactions [10]. There is also a highly conserved BH3 domain (residues 1976–1990) that provides networking interactions for the pro- and anti-apoptotic members of the Bcl-2 family of proteins and two domains of unknown function named ARLD1 (104–374) and ARLD2 (424–815) for *A*rmadillo *r*epeat-*l*ike *d*omains [11]. Although the domain organization of Mule is well determined, the biological functions these domains are involved in are still unknown and more work is required in this direction.

Consistent with complex domain organisation and the protein size, the Mule E3 ligase has been shown to regulate the stability of multiple substrates, thus playing a role in various physiologically important processes, such as cell proliferation, DNA replication and DNA repair. A truncated version of the Mule protein (named UREB1) was originally shown to bind the p53 tumor suppressor protein and to inhibit its transactivation [12,13]. Only relatively recently has the full length Mule protein been first identified by two independent research groups. Mule was shown to be involved in the functioning of the ARF tumor suppressor and consequently named *ARF b*inding *p*rotein *1* (ARF-BP1) [2], and was also found to play a role in the regulation of the Mcl-1 anti-apoptotic protein through ubiquitylation and was consequently named Mule after *Mcl-1 u*biquitin *l*igase *E3* [11]. Other substrates of Mule have since been discovered and include histones [14], the c-Myc [15] and N-Myc [16] oncoproteins, the Cdc6 component of pre-replication complexes[17], TopBP1 topoisomerase II binding protein[18], DNA polymerase β [19], Miz1 transcription factor [20] and the Rev-erbα heme receptor [21].

2. Mule functions in regulation of cell cycle progression

p53 plays a central role in the regulation of the cellular DNA damage response [22–24]. In unstressed cells the level of p53 is very low since it is down-regulated, mainly through Mdm2 ubiquitylation and consequent proteasomal degradation [22,25–28]. DNA damage induces accumulation of the Mdm2 inhibitor ARF (also known as p14 in human cells and p19 in mouse cells) that consequently leads to p53 accumulation and activation of the DNA damage response [29]. However, it was also noted that ARF plays a p53-independent tumor suppressor function since it can also induce proliferation delay in cells that lack a functional p53 or p21 [30–32]. In an attempt to investigate the p53-independent role of ARF, Wei Gu's laboratory first over-expressed and then pulled down tagged ARF from mammalian cells and mass spectrometry analysis of the proteins identified Mule as a major ARF binding partner [2]. Since it is known that ARF regulates p53 levels, they suggested that Mule is a mediator of the ARF effect on p53. They further demonstrated that Mule directly ubiquitylates the p53 protein and thus promotes its proteasomal degradation and that ARF is a strong inhibitor of Mule ubiquitylation activity [2]. This is a very important finding that mechanistically links Mule to the DNA damage response.

3. The role of Mule in DNA repair and maintenance of genome stability

Intriguingly, besides the role in the regulation of p53 levels, Mule was also identified as a major player involved in regulation of base excision repair (BER) proteins. BER enzymes are involved in the processing of a wide spectrum of endogenous and exogenous DNA lesions, including sites of base loss, non-bulky base lesions and DNA single strand breaks (SSBs) of different complexity [33] and alterations in BER lead to genomic instability and cancer (reviewed in [34]). BER is initiated by damage specific DNA glycosylases that recognize and release the corrupted base by hydrolysis of the N-glycosylic bond linking the DNA base to the sugar phosphate backbone. The arising abasic site (AP-site) is further processed by AP-endonuclease 1 that cleaves the phosphodiester bond 5′- to the AP-site, generating a SSB with a 5′-sugar phosphate. This SSB is then repaired by a DNA repair complex that includes DNA polymerase β (Pol β), XRCC1 and DNA ligase IIIα (Lig 3). Pol β possesses AP lyase activity that removes the 5′-sugar phosphate and also, functioning as a DNA polymerase, adds one nucleotide to the 3′-end of the arising single-nucleotide gap. Finally, Lig 3 seals the DNA ends, therefore accomplishing DNA repair (reviewed in [33]). This pathway is commonly referred to as the short patch BER pathway, through which human cells are accomplishing the majority of BER [35,36].

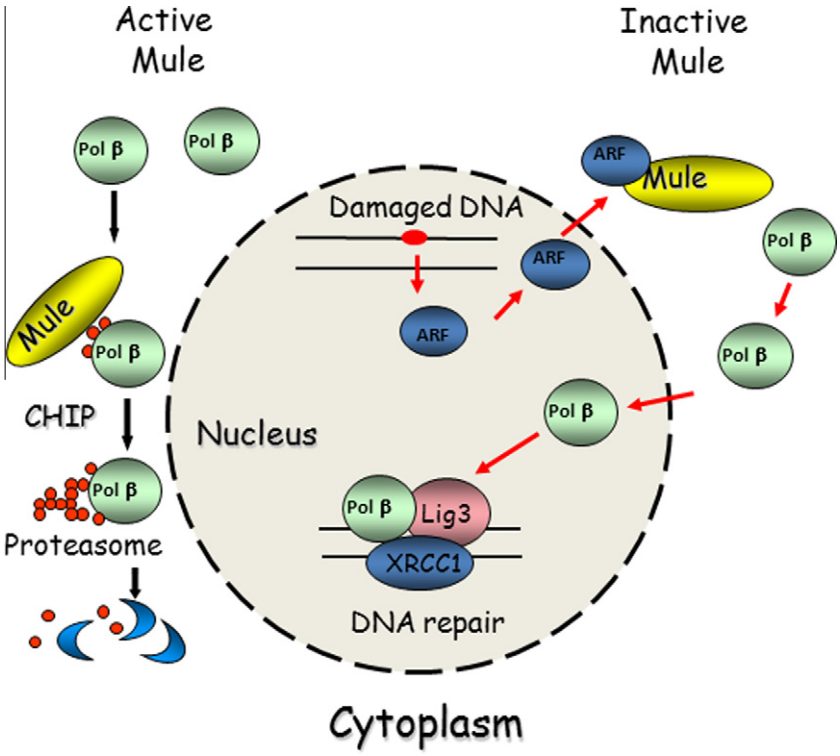

Fig. 2. Regulation of BER by Mule and ARF. If not required for DNA repair, Pol β is ubiquitylated by Mule that is then a target for CHIP mediated polyubiquitylation and subsequent degradation by the proteasome (left side of scheme). However after detection of DNA damage, ARF is accumulated and it inhibits the activity of Mule, thus reducing Pol β degradation and up regulating DNA repair (right side of scheme). The repair of DNA damage will also result in a decreased release of ARF, with a concomitant increased activity of Mule that will down regulate Pol β levels. A new adjustment cycle will therefore begin on the detection of increased levels of DNA damage.

We have recently demonstrated that Mule is playing an important role in the regulation of the cellular levels of Pol β, the central component of the BER pathway [19]. Whilst searching for an E3 ubiquitin ligase responsible for regulation of Pol β levels, we purified both the Mule and CHIP E3 ubiquitin ligases as the major E3 activities ubiquitylating Pol β in human cells. We found that Mule protein is responsible for monoubiquitylation of Pol β that is further targeted for proteasomal degradation via polyubiquitylation by CHIP [19]. Consistent with an earlier finding on the regulation of p53 by Mule [2], we showed that an siRNA knockdown of Mule led to an elevation of Pol β protein levels. Moreover, Mule-knockdown cells repair H_2O_2-induced DNA damage significantly faster than control cells, and the effect of ARF knockdown was entirely the opposite, confirming that ARF is mediating Mule activity in response to DNA damage [19].

We have proposed that since endogenous DNA damage is always occurring, the steady state levels of Pol β required for efficient DNA repair are regulated by Mule ubiquitylation, which is controlled by DNA damage through the release of ARF (Fig. 2). In unstressed cells at low levels of endogenous DNA damage, there is no requirement for high levels of Pol β, which is why the vast majority of newly synthesized Pol β is degraded by Mule and CHIP (Fig. 2, Active Mule). As long as DNA damage is detected, ARF, whose protein levels in unstressed cells are very low [37], is accumulated [38] that inhibits Mule activity. This in turn will lead to a reduced rate of Pol β monoubiquitylation by Mule and consequently a reduced CHIP promoted degradation of Pol β (Fig. 2, Inactive Mule). The concomitant accumulation of Pol β will lead to increased DNA damage repair, as it has been previously demonstrated that resistance to DNA damaging agents correlates with the cellular level of Pol β [39]. Activation of DNA repair will result in a reduced level of DNA lesions, reduced release of ARF, activation of Mule and ubiquitylation of Pol β. This cycle is repeated constantly to adjust the repair capability of cells with the amount of DNA damage. Following acute DNA damage, a sharp accumulation of ARF will lead to the inhibition of Mule activity and thus elevated levels of Pol β in the cytoplasm where Mule is mainly localized [40]. The consequent accumulation of Pol β in the nucleus enables to efficiently repair the amount of excessive DNA lesions.

4. Concluding remarks

Although data on the role of ARF and Mule in controlling DNA replication timing and mediating BER are fragmented, a very interesting picture on the role of these two proteins in coordinating BER and DNA replication is emerging (Fig. 3). We propose that the amount of ARF released in response to DNA damage is proportional to the extent of the DNA damage. This would lead to a quantifiable increase of DNA repair by inhibiting Mule activity and at the same time would cause the proportional delay in initiation of replication by inhibiting the major p53 suppressors, Mule and Mdm2. This would allow enough time to accomplish DNA repair before replication starts. This regulatory loop is self-adjusted since a drop in DNA damage through DNA repair would result in a reduction in the amount of ARF, activation of Mule and Mdm2 and resumption of replication.

Considering the significant impact that Mule has on the stability of DNA repair proteins as well as transcription factors, many researchers have proposed that Mule protein levels may be different in cancer cells compared to normal tissues. Indeed, it was found that Mule expression levels are elevated in a high percentage of tumor cell lines. Tests of various primary colon cancer cells derived from different patients have shown that levels of Mule mRNA correlate with the colon cancer development stage, i.e., almost no

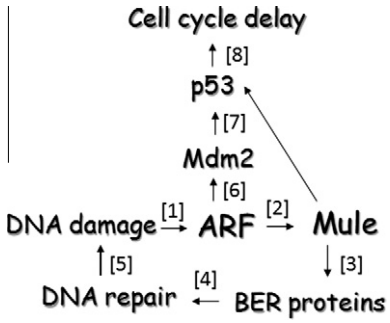

Fig. 3. Mule and ARF coordinate base excision repair and replication. BER and DNA replication delay are regulated by the same proteins. Detection of DNA damage results in accumulation of ARF [1], which activates two cellular processes. By inhibiting Mule it stabilises BER proteins [3] and activates DNA repair [4]. At the same time, inhibition of Mule [2] and Mdm2 [6] lead to an accumulation of p53 [7] and results in cell cycle delay [8]. After DNA repair is accomplished [5], the reduction of DNA damage initiates a reverse cycle by reducing DNA repair and releasing the cell for replication.

Mule mRNA was detected in normal cells whereas in adenomas and adenocarcinomas the gene expression was elevated by approximately 30% and 50%, respectively. In addition, it was also shown that Mule mRNA levels are comparatively high in many lung and breast [15], as well as in cervix, stomach and biliary duct cancer cell lines [41]. The importance of the *mule* gene in colon cancer development was also confirmed by Yoon et al. as expression of this gene was found to be at a moderate or high level in approximately 40% of colon carcinoma tissues (samples from 70 patients were tested) compared to non-tumor colon. Importantly, Mule and p53 levels were found to inversely correlate in the vast majority of colon cancer tissues tested [41]. Furthermore, Chen and co-authors [42] were able to show that Mule protein depletion represses proliferation of breast cancer cell lines characterized by mutations in the p53 gene.

We speculate that overexpression of Mule in some cancer cells might be the primary reason for carcinogenesis progression because it does deregulate p53 levels and consequently abolishes p53-dependent responses to genotoxic stress. This model predicts that inhibition of Mule in such cells should have a therapeutic effect.

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