

Regulation of Apoptosis and Cytokinesis by the Anti-apoptotic E2/E3 Ubiquitin-Ligase BRUCE

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Abstract. Members of the inhibitor of apoptosis protein (IAP) family are key regulators of apoptosis as they bind and inhibit caspases and other pro-apoptotic factors. Recent findings suggest that these proteins play additional roles, e.g., in cell cycle regulation, angiogenesis, and carcinogenesis. Here, we review the function of BRUCE (BIR repeat-containing ubiquitin-conjugating enzyme), an unusual 528-kDa IAP with ubiquitin ligase activity, and describe its role in apoptosis and cytokinesis. Additionally, we discuss how these seemingly unrelated functions might be linked.

1 Apoptosis Regulation by Inhibitor of Apoptosis Proteins

Programmed cell death, or apoptosis, is driven by proteases of the caspase family, which initiate and execute cell death by cleaving crucial cellular proteins (Salvesen and Duckett 2002). Caspases can be inhibited by so-called BIRPs, proteins that harbor baculovirus inhibitor of apoptosis repeats (BIRs). BIRPs also protect cells from apoptosis by ubiquitylation and degradation of pro-apoptotic factors (Verhagen et al. 2001). The BIR domain represents a zinc-binding fold of approximately 70 amino acids harboring a CX₂CX₆WX₃DX₅HX₆C consensus sequence (Hinds et al. 1999). Notably, linker sequences at the boundaries of BIR domains have been shown to bind to activated caspases, thereby sterically preventing access to substrates (Riedl et al. 2001; Huang et al. 2001; Chai et al. 2001).

In mammalian cells, after loss of mitochondrial integrity, apoptosis-inducing factors such as Smac and the serine protease HtrA2 are released through permeability transition pores. These factors can bind to BIRPs via a short N-terminal sequence on the same surfaces where caspases bind. This results in a competition of Smac/HtrA2 and caspases for BIRP binding. Therefore, as soon as the cytoplasmic concentration of Smac/HtrA2 rises above a critical level, caspases are relieved from repression by BIRPs and can initiate apoptosis (Ditzel and Meier 2002).

2 BRUCE Is a Cell Death Regulator

BIRPs of the IAP class usually contain several BIR domains and a C-terminal RING finger domain, which endows the protein with E3 ubiquitin ligase activity. BRUCE is an unusual BIRP/IAP because of its enormous size (528 kDa) and the presence of a single N-terminally located BIR domain and a C-terminally located ubiquitin-conjugating enzyme (UBC) domain. BRUCE was discovered in a screen using degenerate primers for ubiquitin-conjugating (UBC) enzymes (the E2 enzymes of the ubiquitin-conjugation pathway) (Hauser 1992). In vitro BRUCE can form a thioester with ubiquitin and can transfer ubiquitin to substrate proteins, demonstrating that it functions as a chimeric

E2/E3 ubiquitin ligase (Hauser et al. 1998; Bartke et al. 2004; Hao et al. 2004). Cell fractionation and membrane flotation experiments revealed that BRUCE is a peripheral, membrane-associated protein (Bardroff 1997). Furthermore, BRUCE was found to localize mainly to the TGN and perinuclear vesicles. It was also reported that cancer cells expressing high levels of BRUCE are resistant to apoptosis-inducing agents, and that conversely downregulation of BRUCE results in sensitivity to these agents (Chen et al. 1999).

The anti-apoptotic function of BRUCE is well studied in *Drosophila*. The BRUCE homolog, dBRUCE, was found to inhibit apoptosis when executed by the Smac/HtrA2-related pro-apoptotic factors Reaper and Grim, but not Hid (Vernooy et al. 2002), suggesting that dBRUCE plays a more specialized role in cell death regulation. Indeed, dBRUCE is crucial for the regulation of the concluding step of spermatogenesis in *Drosophila* when spermatids remove their bulk cytoplasm during spermatid individualization. This process is accompanied by an apoptosis-like caspase activation, which has to be locally and temporally restricted (Arama et al. 2003). Strikingly, in *dbruce*^{-/-} flies, which are male sterile, spermatids acquire hypercondensed nuclei and finally degenerate, indicating uncontrolled apoptosis. During spermatid individualization dBRUCE was shown to interact with a testis-specific multisubunit ubiquitin ligase complex (Arama et al. 2007). dBRUCE directly binds to the substrate-recruiting subunit Khl10 of this ligase complex composed of Cullin-3 and Roc1b. Notably, the Cul3/Roc1b/Khl10 complex is required for the transient caspase activation in fly testis, and therefore probably also for dBRUCE degradation.

In mammalian cells, BRUCE functions as a typical inhibitor of apoptosis protein (Bartke et al. 2004; Hao et al. 2004). It can bind and inhibit activated initiator caspases-8 and -9 and executioner caspases-3, -6, and -7. Furthermore, both Smac and HtrA2 are able to compete for BRUCE-bound caspases. Interestingly, BRUCE is also a substrate of caspases and the serine protease HtrA2, pointing to a role in regulating apoptosis at early stages when proteolytic activity mediated by these enzymes is still low. Furthermore, BRUCE can ubiquitylate both Smac and HtrA2 and probably also caspase-9, a caspase initiating apoptosis after mitochondrial permeabilization (Bartke et al. 2004; Hao et al. 2004). Although only a monoubiquitylation on the substrate Smac was observed

in vitro, BRUCE might collaborate with other E3 ligases for polyubiquitylation and proteasomal degradation. This is likely also the case for other BRUCE substrates such as HtrA2 or caspases. However, since BRUCE is localized in interphase cells mainly to the TGN in interphase cells and endosomes, its anti-apoptotic activity is most likely restricted to these sites (Hauser et al. 1998). Notably, BRUCE is also downregulated by the ubiquitin-proteasome system. Nrdp1/FLRF, a RING finger-containing ubiquitin ligase initially found to be involved in the degradation of the receptor tyrosine kinases ErbB3/4 (Diamonti et al. 2002; Qiu and Goldberg 2002), can act as a ubiquitin ligase for BRUCE, leading to its proteasome-dependent degradation (Qiu et al. 2004). Nrdp1 also interacts with a deubiquitylating enzyme USP8 (UBPY) (Wu et al. 2004), a cysteine protease with the highest similarity to yeast Doa4. USP8 is implicated in cell cycle regulation, downregulation of the EGF receptor, and the regulation of the ESCRT (endosomal sorting complex required for transport) components Hrs and STAM (Clague and Urbé, 2006). Depletion of USP8 leads to an increase in the size and number of multivesicular bodies (MVBs) and an accumulation of ubiquitin on their surface (Row et al. 2006). Figure 1 illustrates the interaction of BRUCE with some of its binding partners.

3 Regulation of Cytokinesis

Besides apoptosis regulation, some BIRPs such as survivin (Li et al. 1999) and cIAP1 (Samuel et al. 2005) also participate in cell cycle events and cytokinesis. Survivin is a 17-kDa protein, which harbors an N-terminal BIR domain (which resembles the BIR of BRUCE) and a C-terminal coiled-coil domain. It is a core component of the chromosomal passenger complex and is hence essential for cytokinesis. Similar to BRUCE, survivin can firmly associate with Smac but shows only limited potential in inhibiting caspases in vivo (Song et al. 2003; Banks et al. 2000).

cIAP1, a predominantly nuclear protein in interphase, has recently been shown to participate in cell cycle regulation as well (Samuel et al. 2005). After nuclear reaccumulation in telophase, a small pool of cIAP1 associates with the midbody in a complex with survivin. Cells over-

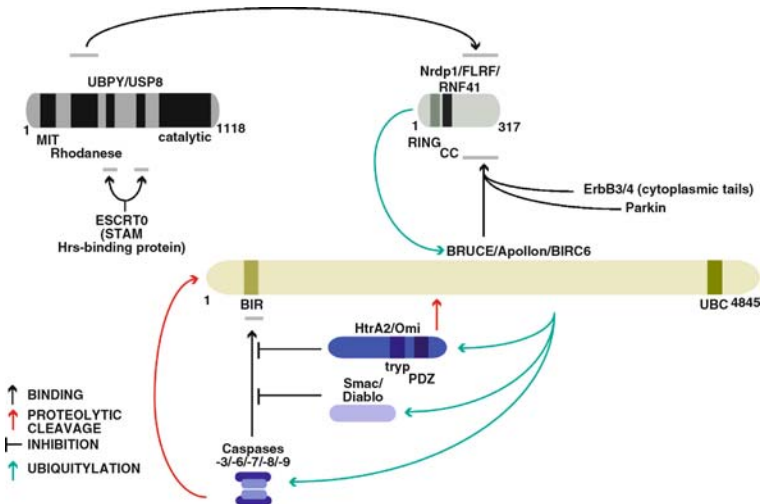


Fig. 1. Protein interaction network of BRUCE. Schematic diagram depicting the multifunctional BRUCE (alias Apollon or BIRC6), the RING E3-ligase Nrdp1 (alias FLRF or RNF41), and the de-ubiquitylating enzyme USP8 (alias UBPY). The domain architectures and sizes (in amino acids) of the proteins are shown. Protein–protein interactions are indicated. *tryp* trypsin domain, *CC* coiled-coil, *MIT* domain in microtubule interacting and trafficking proteins, *BIR* baculovirus inhibitor of apoptosis repeat, *UBC* ubiquitin conjugating enzyme domain, *ESCRT* endosomal sorting complex required for transport

expressing cIAP1 exhibit an accumulation in G2/M, grow slower, and exhibit cytokinesis defects.

Interestingly, BRUCE-knockout mice die perinatally not because of apoptosis induction, but rather impaired placental development, which can be attributed to insufficient differentiation (Lotz et al. 2004). Indeed, we recently discovered that BRUCE, besides its role in apoptosis regulation, is a novel player of cytokinesis and central for abscission (Pohl and Jentsch 2008).

Cytokinesis is the final step of cell division in which daughter cells physically separate. The earliest discernible event during this process is the formation of a cortical actomyosin ring. Constriction of this ring leads to furrowing that generates a narrow intercellular bridge. Con-

comitantly with furrow ingression, another cytokinesis-specific structure, the midbody, assembles by bundling of spindle-midzone microtubules. In the midpoint of the intercellular bridge that results from furrowing, midbody microtubules embrace a phase-dense circular structure called the midbody ring (also called the Flemming body). Finally, during abscission, the tubular bridge is cleaved and two daughter cells are formed.

Recent work demonstrated that the midbody serves as a rigid but dynamic platform on which different processes that drive cytokinesis converge, e.g., kinase signaling, degradation of cell cycle regulators, rearrangements of membranes and of the cytoskeleton. It became clear that traffic-regulating GTPases, such as Arf1, Arf6, and Rab11, play an important role in cytokinesis (Albertson et al. 2005). These proteins seem to cooperate with the multisubunit exocyst membrane-targeting complex to deliver endosomal vesicles to the site of abscission. Interestingly, a proportion of vesicles seem to arrive at the midbody ring chiefly from only one of the prospective daughter cells (Gromley et al. 2005), suggesting an intrinsic asymmetric element in cytokinesis. Besides these proteins, only a few additional components required for membrane remodeling at the midbody are currently known. One such factor is centriolin, a protein that binds to the maternal centriole and is needed for the proper localization of the exocyst complex to the midbody ring (Gromley et al. 2005).

Previous studies also point to a role of ubiquitylation and proteasomal activity in cytokinesis regulation (Pines 2006). Notably, components of the ubiquitin–proteasome system are concentrated at midbodies (Grenfell et al. 1994; Wojcik et al. 1995), and several proteins that are crucial regulators of mitosis (e.g., the chromosomal passenger proteins aurora B and survivin, Polo-like kinase 1 (Plk1), and the actin-associated protein anillin) are degraded during cytokinesis (Pines 2006). Hence, the activity of the proteasome also seems to be important after anaphase onset. Interestingly, combined inhibition of Cdk1 and proteasomes with a subsequent release of inhibition in late cytokinesis can revert cells into a presumably pre-anaphase state (Potapova et al. 2006). Since the ubiquitin-controlled ESCRT pathway is also necessary for abscission (Carlton and Martin-Serrano 2007), nonproteolytic functions of ubiquitin play a role in late cytokinesis as well.

4 Final Stages of Cytokinesis Are Controlled by BRUCE

During cytokinesis, BRUCE localizes to the midbody ring where it binds mitotic regulators and components of the vesicle-targeting machinery (Pohl and Jentsch 2008). Localization to the midbody ring depends on a targeting domain in BRUCE (MTD) that comprises approximately 150 amino acids. This domain can mediate direct interactions with the midbody ring resident mitotic kinesin-like protein 1 (MKLP1). Analysis of cells that overexpress this targeting domain (which acts as a dominant-negative BRUCE construct specific for this localization), or cells that were treated with BRUCE-specific siRNAs, revealed that BRUCE is involved in the delivery of membranes to the site of abscission. Thus, the phenotypes that occur upon BRUCE depletion are most likely caused by a failure of membrane delivery and defective recruitment of mitotic regulators to the midbody ring. Notably, these phenotypes resemble those caused by centriolin depletion (Gromley et al. 2005; see above).

Several regulators of vesicular trafficking associate with the N-terminal region of BRUCE, including the GTPases Rab8/Rab11 and components of the exocyst (Fig. 2). Notably, BRUCE relocates during cell division, which appears to be driven by vesicle movements along microtubules. The majority of these vesicular structures where BRUCE localizes are large pleiomorphic traffic intermediates (Peränen et al. 1996; Urbé et al. 1993). In interphase cells, BRUCE localizes to the TGN and tubular/recycling endosomes, but during cytokinesis, a fraction travels to the midzone where it arrives specifically at the midbody. Notably, BRUCE associates with the midbody ring concomitant with its appearance in telophase, travels after completed abscission into one daughter cell together with the midbody ring, and remains bound to the discarded midbody ring until it dissolves.

BRUCE depletion also leads to cytokinesis-coupled apoptosis and the formation of elongated syncytia. Remarkably, in HeLa cells, BRUCE depletion leads to apoptosis precisely when the dividing cells attempt abscission. It should be noted, however, that BRUCE depletion in other cell types than HeLa did not induce cytokinesis-associated apoptosis but variable failures in cytokinesis. It is thus likely that cytokinesis-associated apoptosis might be restricted to cell types that require

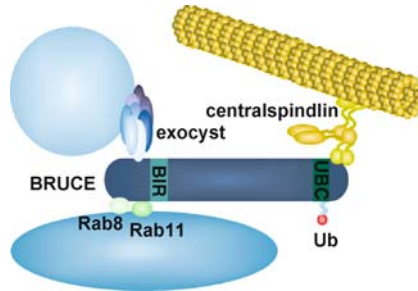


Fig. 2. BRUCE as a protein-binding platform at the midbody ring. BRUCE physically links membrane targeting and midbody ring components. In addition, it might also ubiquitylate proteins of the midbody ring. The complex of MKLP1 and MgcRacGAP is described as centralspindlin. *BIR* baculovirus inhibitor of apoptosis repeat, *UBC* ubiquitin-conjugating enzyme domain, *Ub* ubiquitin

specialized IAPs or a balanced ratio of different IAPs during cytokinesis such as HeLa cells (Crnkovic-Mertens et al. 2006). Indeed, BRUCE is part of a mixed BIRP/IAP complex with survivin, suggesting that they cooperate in apoptosis regulation. BRUCE not only co-localizes with survivin at the midzone and physically interacts with this BIRP, but also monoubiquitylates the protein *in vitro*. This suggests that BRUCE might trigger survivin degradation or that ubiquitylation might control midbody dynamics.

Intriguingly, cytokinesis is accompanied by a dramatic relocation of ubiquitin. Ubiquitin first appears as striking ball-like accumulations flanking the midbody ring symmetrically. Then, after a period of perceptible absence, ubiquitin reappears directly on the midbody ring (Pohl and Jentsch 2008). Fluorescence redistribution after photobleaching (FRAP) experiments showed that ubiquitin on the midbody ring is very static, suggesting that midbody ring ubiquitylation occurs only once per cell cycle, possibly because it plays a structural role. The crucial substrates for ubiquitylation are currently unknown, but BRUCE and MKLP1 are ubiquitylated *in vivo*. Moreover, USP8, which associates with BRUCE and the midbody ring, may function as their deubiquitylating enzyme. Remarkably, partial BRUCE depletion interferes with the ubiquitin dynamics at the midbody (Pohl and Jentsch

2008), suggesting that BRUCE may directly participate in the observed ubiquitylation events.

5 Conclusion

The midbody ring represents a unique cellular structure (about 1.5 μm in diameter) which requires several processes for abscission to converge. BRUCE appears to be an integral part of the ring and is required for its normal formation and integrity. Since the midbody ring is densely ubiquitylated and BRUCE possesses E2/E3 ubiquitin ligase activity (Bartke et al. 2004), it seems likely that BRUCE's ubiquitylation activity is directly involved in midbody ring function or integrity. Furthermore, BRUCE also functions as an anti-apoptotic IAP, suggesting that the observed cytokinesis-associated apoptosis of BRUCE-depleted HeLa cells is a direct consequence of its absence. This suggests that BRUCE, through the combination of different activities and binding sites, is ideally suited to coordinate multiple functions during cytokinesis. It remains to be seen how this "molecular Swiss army knife" coordinates cytokinesis events at the molecular level and what the crucial substrates are.

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