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E2F1 interacts with BCL-xL and regulates its subcellular localization dynamics to trigger cell death

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Abstract

E2F1 is the main pro-apoptotic effector of the pRB-regulated tumor suppressor pathway by promoting the transcription of various pro-apoptotic proteins. We report here that E2F1 partly localizes to mitochondria, where it favors mitochondrial outer membrane permeabilization. E2F1 interacts with BCL-xL independently from its BH3 binding interface and induces a stabilization of BCL-xL at mitochondrial membranes. This prevents efficient control of BCL-xL over its binding partners, in particular over BAK resulting in the induction of cell death. We thus identify a new, non-BH3-binding regulator of BCL-xL localization dynamics that influences its anti-apoptotic activity.

Keywords apoptosis; BCL-2 family; BCL-xL mobility; E2F1

Subject Category Autophagy & Cell Death

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Introduction

Major tumor suppressor pathways, such as those relying on p53 or pRB/E2F1, promote pro-apoptotic signals that ultimately converge on mitochondrial outer membrane permeabilization (MOMP) [1]. BCL-2 (B-cell lymphoma/leukemia-2) family proteins are critical regulators of this process [2–4]. They are classified into three functionally distinct subgroups depending on their BCL-2 homology (BH) domain composition: multidomain anti-apoptotic proteins (BCL-xL, BCL-2, MCL-1...), oppose multidomain pro-apoptotic proteins (BAX, BAK), and their upstream effectors, the BH3-only

pro-apoptotic members (BAD, BIM, BID, NOXA, PUMA...) [5]. They do so in great part by engaging in a network of physical interactions, in which the BH3 domain of pro-apoptotic proteins is bound to the hydrophobic groove at the surface of anti-apoptotic proteins. The balance between these different interactions determines whether or not MOMP occurs due to BAX/BAK oligomerization in mitochondrial membranes.

Changes in BCL-2 protein complexes that lead to MOMP in response to tumor suppression have been extensively described. Not only p53 transcriptionally regulates the expression of BH3-only proteins but also it acts through a non-transcriptional effect: It indeed localizes to mitochondria [6] where it can interact with anti-apoptotic BCL-2 proteins [7] or with BAX to directly activate it [8]. Therefore, p53 exerts a widespread effect on mitochondrial apoptotic priming by impacting, in many ways, on the composition and assembly of the BCL-2 network [9]. Several BCL-2 proteins including BAK and BCL-xL localize preferentially at intracellular membranes (especially in the outer mitochondrial membrane) due to a hydrophobic C-terminal anchor [10]. The current view is that competence to die can be inferred by snapshot analysis of the state of the BCL-2 network at mitochondria [11]. However, recent data have highlighted that more dynamic features may also intervene. At a whole cell level, BAX, BAK, and BCL-xL are not only targeted to mitochondria but their outer membrane-associated and integral forms are also shuttled back (“retrotranslocated”) at varying rates [12]. Retrotranslocation of pro-apoptotic proteins protects from cell death [13–15]. In contrast, the mechanisms of BCL-xL retrotranslocation and its impact on MOMP onset are not yet completely understood.

E2F1 is the main pro-apoptotic effector of the pRB-regulated tumor suppressor pathway. It was described to promote p53-dependent and p53-independent apoptosis in response to either

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oncogenic stress or DNA damage [1]. E2F1 is recognized to mainly function as a transcription factor, inducing the expression of numerous pro-apoptotic actors, including some BH3-only proteins such as PUMA and BIM. Its transcriptional activity is negatively regulated by pRB in most cases, even though positive modulation was reported upon genotoxic and apoptotic stresses [16,17]. Because some reports hinted that E2F1 might also promote apoptosis by transcription-independent mechanisms [18,19], we herein investigated whether it might exert a direct effect on the mitochondrial BCL-2 network, as was reported for p53 and for its binding partner pRB [7,20]. We herein show that E2F1 physically interacts with BCL-xL and that it inhibits BCL-xL localization dynamics, which we establish here as critical for negative regulation of MOMP.

Results and Discussion

E2F1 pro-apoptotic activity relies on its stabilization at the protein level in response to cell death stimuli, such as DNA damage, resulting in numerous post-translational modifications [17]. Consistent with this, treatment of pRB-deficient, p53-null Saos-2 cells with the genotoxic agent etoposide, at a concentration that induced apoptosis, enhanced E2F1 expression (Fig 1A). E2F1 contributed to cell death induction, since downregulation of its expression by siRNA significantly decreased etoposide-induced cell death rates (Fig 1B). Subcellular fractionation Saos-2 cells (Fig 1C) and of other cell types (Fig EV1A), based on differential centrifugations, revealed the presence of endogenous E2F1 in the heavy membrane fraction that includes mitochondrial and reticulum endoplasmic markers. Mitochondrial fractions with reduced endoplasmic reticulum markers obtained by a second approach based on magnetically labeled anti-TOM22 antibody showed enriched E2F1, further highlighting its mitochondrial localization (Fig 1C).

We investigated whether E2F1 would contribute to apoptosis when localized at intracellular membranes, and specifically at mitochondria. We engineered a mitochondrial-targeted, GFP-fused wild-type E2F1 (GFP-E2F1, hereinafter named wt form) to which was fused the mitochondrial targeting sequence of ornithine carbamoyl-transferase (OTC-GFP-E2F1, hereinafter named OTC form), using a strategy previously used to investigate the mitochondrial effects of p53 and pRB [6,20] (Fig EV1B and Appendix Fig S1A). Specific mitochondrial targeting of OTC-GFP-E2F1 was confirmed by fluorescence microscopy (Fig EV1C). We investigated pRB- and p53-independent biological effects of mitochondrial-targeted E2F1 by transient transfection of Saos-2 cells followed by investigation of GFP-positive cells. As shown in Fig 1D, enhanced expression of either wt or OTC forms was sufficient *per se* to trigger cell death. Importantly, both E2F1 forms sensitized Saos-2 cells to etoposide-induced cell death, strongly arguing that mitochondrial E2F1 potentially contributes to cell death onset (Fig 1D). As previously published [6], targeting GFP to mitochondria using OTC did not induce cell death (Fig EV1D). Ectopic expression of wt or OTC E2F1 forms induced caspase-3 activation and triggered caspase-dependent cell death since the pan-caspase inhibitor Q-VD-OPh completely protected cells (Fig EV2A and B). To directly investigate whether enhanced E2F1 expression triggers MOMP, we used the reporter breast cancer cell line MDA-MB231 that stably expresses an OMI red fluorescent fusion protein which is degraded by the proteasome

when released from mitochondria following MOMP [21] (Fig EV2C). Quantitative assays by cytometry based on red fluorescence intensity of mitochondria allowed us to discriminate, among GFP-positive cells, intact cells from cells that underwent MOMP (Fig EV2D). Both wt and OTC forms triggered MOMP (as detected by a decrease in red fluorescence intensity of mitochondria) in these cells and Annexin V staining (Figs 1E and EV2E).

As expected, transient transfection of OTC-GFP-E2F1 had no detectable effect on mRNA expression of E2F1 canonical pro-apoptotic transcriptional targets such as TP73, PUMA/BBC3, or BIM/BCL2L11, whose expression was induced by GFP-E2F1 in control experiments (Fig EV2F). In addition, it had no impact on HRK expression, which was reportedly induced by wt E2F1, via the indirect inhibition of a repressor complex [22]. To further substantiate that the apoptotic effects of mitochondrial E2F1 reported above ensue, at least in part, from transcription-independent mechanisms, we used GFP-fused E2F1 E132 (named E132 form), a DNA-binding-defective mutant (Fig EV1B and Appendix Fig S1A) [23]. This E2F1 transcription-deficient form induced MOMP and apoptosis by itself, and it sensitized Saos-2 cells to etoposide treatment (Fig 1D and E). Likewise, a mitochondrial-targeted transcription-deficient E2F1 mutant (OTC-GFP-E132) also induced apoptosis upon overexpression (Fig EV1D).

Downregulation of BAK and/or BAX expression by RNA interference showed that death induced by wt and OTC forms relied preferentially on BAK in Saos-2 cells (Fig 2A and Appendix Fig S1B). This was consistent with the identities of anti-apoptotic proteins that prevented cell death induced by either form of E2F1: ectopic BCL-xL and MCL-1 but not BCL-2 (which does not to modulate BAK-dependent cell death, most likely as a result from its lack of interaction with this pro-apoptotic protein) promoted survival (Fig 2B and Appendix Fig S1C). BH3-binding activity was required for BCL-xL to inhibit E2F1-induced cell death, since the BCL-xL R139D mutant, whose BH3 binding is impaired [24] (see also below), did not protect cells in the same settings. Moreover, treatment with the BH3 mimetic inhibitor WEHI-539 (which specifically targets BCL-xL) reverted the protection afforded by overexpressed BCL-xL against E2F1-induced cell death (Fig 2B). Consistent with above data, both E2F1 form and the E132 form drastically enhanced the pro-apoptotic effects of ectopically expressed BAK (Figs 2C and EV3, and Appendix Fig S1D). Notably, we also observed a similar sensitizing effect of wt and OTC fused E2F1 upon overexpression of BAX and of the upstream activators, the BH3-only proteins BIM, PUMA, and truncated BID (tBID) (Fig 2C and Appendix Fig S1D). Thus, although our data put forth a preferential link between E2F1 and BAK, this may be not exclusive, and BAX may contribute under certain circumstances.

We then investigated whether the non-transcriptional pro-apoptotic impact of E2F1 on BCL-xL-regulated BAK-mediated MOMP relied on a molecular interaction between E2F1 and BCL-xL, which both localize at intracellular membranes and at the mitochondria in particular (Figs 1B and EV1A). As shown in Fig 3A, E2F1 co-immunoprecipitated with BCL-xL in lysates from Saos-2 cells. Importantly, this interaction was detected in numerous other cell lines independently from their pRB and p53 status (Figs 3A and EV4A). Bioluminescence resonance energy transfer (BRET) assays confirmed a specific proximity between Renilla Luciferase fused to E2F1 (RLuc-E2F1) and YFP-BCL-xL at a whole cell level (Fig 3B). In sharp contrast to these observations, we could not detect any BRET signals

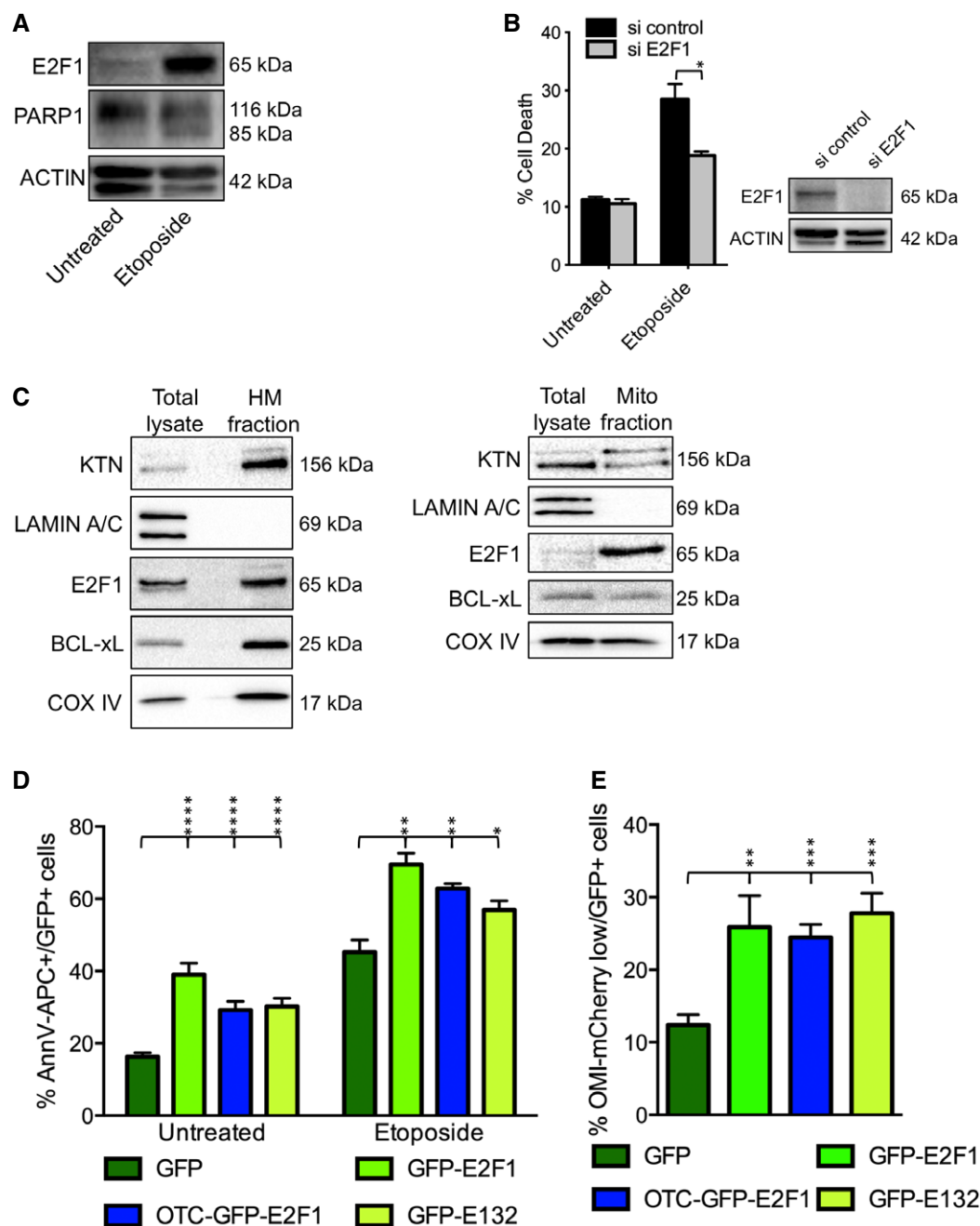


Figure 1. E2F1 localizes to mitochondria in Saos-2 cells, where it promotes apoptosis.

- A** Apoptotic signal induces E2F1 stabilization. Saos-2 cells were treated for 16 h by 50 μ M etoposide or not (untreated) before Western blot analysis of E2F1 expression and PARP1 cleavage.
- B** Etoposide induces apoptosis in E2F1-dependent manner. Saos-2 cells were transfected with control or E2F1 siRNA for 24 h and treated as in (A) before cell death analysis by trypan blue staining. Western blot controlling the E2F1 siRNA extinction is inserted.
- C** E2F1 constitutively localizes to mitochondria. Saos-2 cells were fractionated, and equal amounts of total lysate versus heavy membrane fraction (HM fraction) or mitochondrial-enriched fraction (Mito fraction) were analyzed by Western blot analysis for E2F1 and BCL-xL expression. KTN, LAMIN A/C, and COX IV serve as markers of endoplasmic reticulum, nuclei, and mitochondria, respectively. Data shown are representative of at least three independent experiments.
- D** Ectopic E2F1 expression triggers apoptosis. Saos-2 cells were transfected with the indicated E2F1 expression vectors and treated or not with etoposide (50 μ M) for an additional 48 h. Apoptosis was evaluated by Annexin V-APC staining among GFP-positive cells using flow cytometry analysis.
- E** E2F1 triggers MOMP. MDA-MB231 cells stably expressing OMI-mCherry were transfected with the indicated expression vectors. 48 h post-transfection, MOMP was quantified by determining the mCherry low fluorescence cell percentage among GFP-positive cells using flow cytometry analysis.

Data information: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 versus control (Student's t -test). Graph bars represent the average of at least three independent experiences, and the error bars show the SEM.

Source data are available online for this figure.

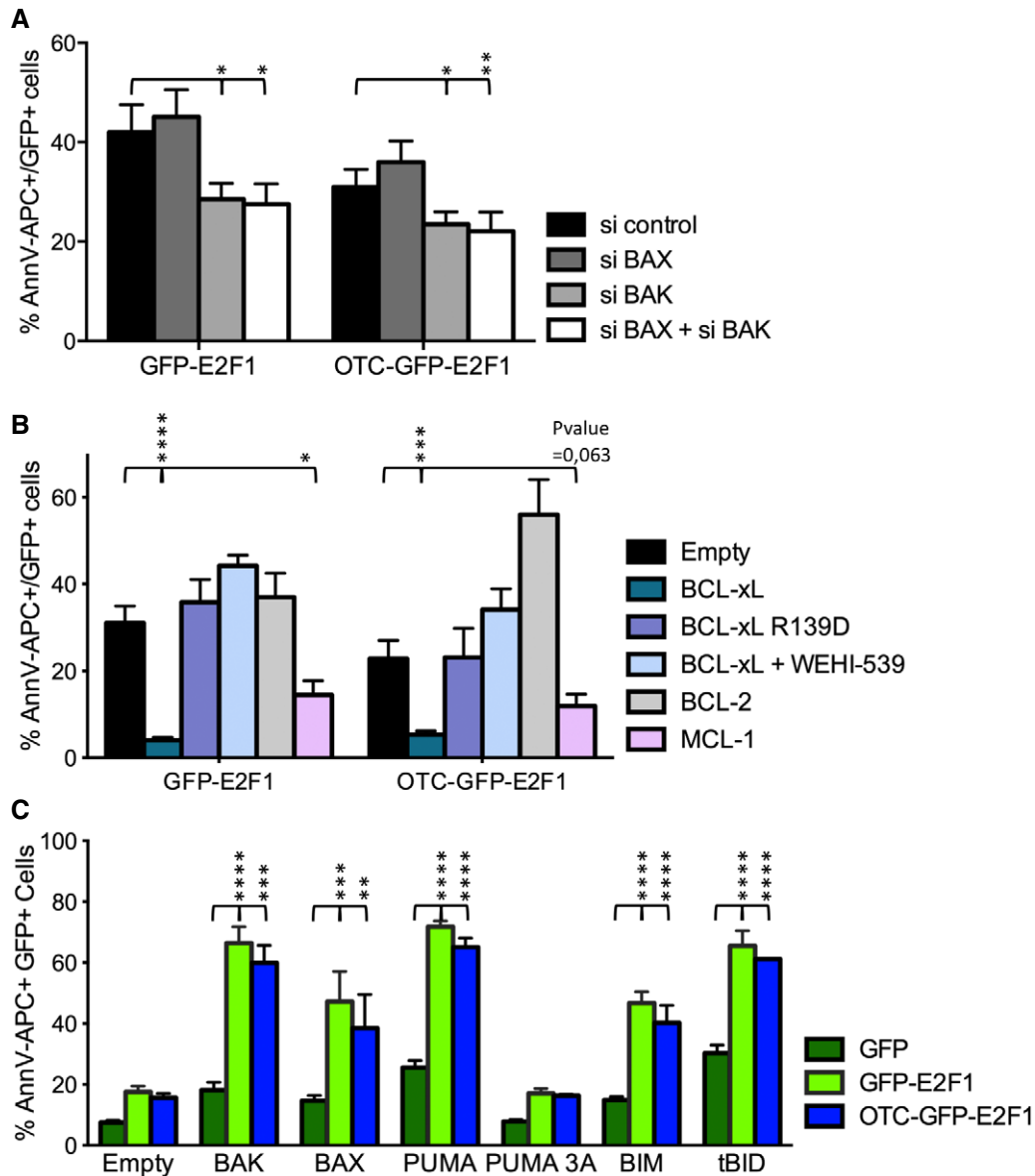


Figure 2. E2F1 promotes mitochondrial apoptosis controlled by the BCL-2 family.

A E2F1 induced apoptosis in a BAK-dependent manner. Saos-2 cells were transfected with control, BAK or BAX siRNAs. 24 h later, cells were transfected with the expression vectors coding for either GFP-E2F1 or OTC-GFP-E2F1 for 48 h before apoptosis measurement as described in Fig 1D.

B BCL-xL suppresses E2F1-induced apoptosis as a BH3-binding protein. Saos-2 cells were co-transfected with plasmids encoding the indicated anti-apoptotic proteins (BCL-xL or its R139D mutant, BCL-xL treated with WEHI-539, BCL-2 or MCL-1) and GFP-E2F1, or OTC-GFP-E2F1 in a molecular ratio 3:1. 24 h later, apoptosis was analyzed as described in Fig 1D.

C Mitochondrial-targeted E2F1 promotes cell death activity induced by BAK, BAX, or BH3-only activators overexpression. Saos-2 cells were co-transfected with the indicated expression vectors and for GFP, GFP-E2F1, or OTC-GFP-E2F1 in a molecular ratio 3:1. 24 h later, apoptosis was analyzed as described in Fig 1D. The deficient activator PUMA 3A, carrying L141A D146A L148A substitutions in the BH3 domain was used as a negative control.

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus control (Student's *t*-test). Graph bars represent the average of at least three independent experiences, and the error bars show the SEM.

between E2F1 and BAK by BRET experiments (Fig EV4B). Moreover, pull-down assays using recombinant GST-fused E2F1 [25] and recombinant-soluble BCL-xL demonstrated a direct interaction between both proteins (Fig 3C). We mapped the minimal domain required for E2F1 to interact with BCL-xL to the DNA-binding domain: Fusion proteins containing this region only (DBD, residues 114–191, Fig EV1B and Appendix Fig S1A) showed BRET signals

with BCL-xL (Fig 3B) and pulled down recombinant BCL-xL (Fig 3C). E2F1 deleted in its C-terminal end (ΔC , Fig EV1B and Appendix Fig S1A) behaved similarly (Fig 3B), but a form deleted in its N-terminal end that encompasses the DBD (ΔN , Fig EV1B and Appendix Fig S1A) showed strongly reduced BRET signals and pull-down properties (Figs 3B and EV4C). Consistent with the notion that its interaction with BCL-xL contributes to E2F1 pro-apoptotic activity,

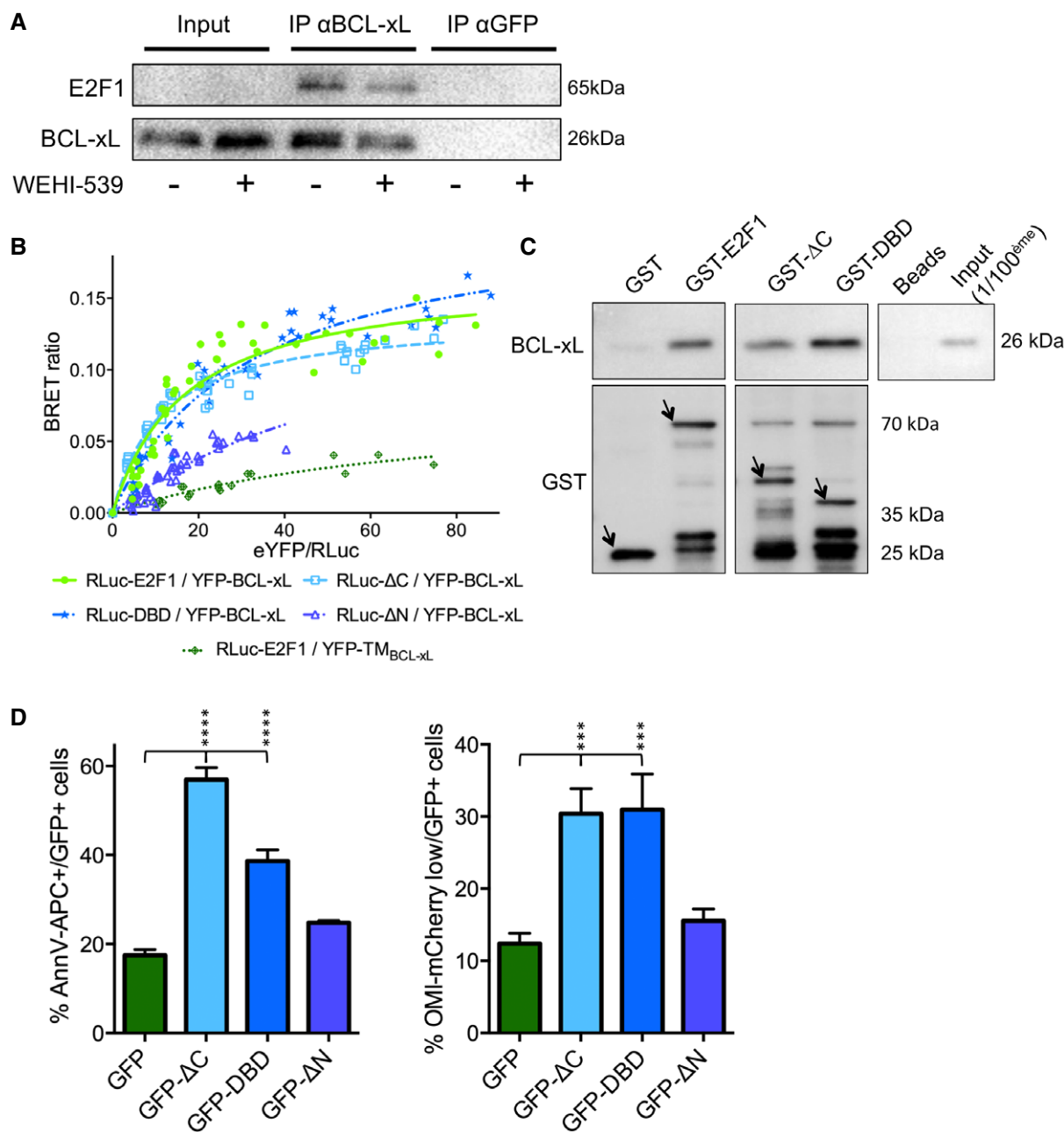


Figure 3. E2F1 interacts with BCL-xL.

- A** E2F1 interacts with BCL-xL in a BH3-mimetic-resistant manner. Endogenous BCL-xL was immune-precipitated from Saos-2 cell extracts treated or not with WEHI-539 (1 μ M for 24 h) with anti-BCL-xL or negative control anti-GFP, and then, endogenous E2F1 association was assessed by Western blotting.
- B** E2F1 and BCL-xL interact in live cells. BRET saturation assay analysis was performed in MCF-7 cells using increasing amount of vectors encoding for YFP-BCL-xL or YFP-TM_{BCL-xL} in the presence of a fixed amount of the vector encoding RLuc-E2F1, RLuc- Δ C, RLuc-DBD, or RLuc- Δ N. BRET ratios are measured for every YFP-BCL-xL plasmid concentrations and are plotted as a function of the ratio of total acceptor fluorescence to donor luminescence. No BRET saturation curve was obtained neither by using RLuc-E2F1 and YFP fused to the C-terminal transmembrane domain of BCL-xL (YFP-TM_{BCL-xL}) demonstrating the specific interaction between E2F1 and BCL-xL, nor by using RLuc- Δ N indicating that N-terminal domain of E2F1 is required to interact with BCL-xL. The data were fitted using a nonlinear regression equation assuming a single binding site. Data are representative of at least three independent experiments.
- C** E2F1 DNA-binding domain interacts with BCL-xL as recombinant proteins. GST pull-down analysis was performed using recombinant GST-E2F1, GST- Δ C, GST-DBD, and purified BCL-xL proteins.
- D** E2F1 DNA-binding domain is sufficient to induce mitochondrial apoptosis. Saos-2- and MDA-MB231-expressing OMI-mCherry cells were transfected with the indicated expression vectors. 48 h later, apoptosis (left panel) and MOMP among GFP-positive cells (right panel) were analyzed as described in Fig 1D and E.

Data information: *** P < 0.001, **** P < 0.0001 versus control (Student's t -test). Graph bars represent the average of at least three independent experiences, and the error bars show the SEM.

Source data are available online for this figure.

and in agreement with preceding results [18], ectopic expression of the DBD and ΔC forms, but not of the ΔN one, induced cell death, MOMP, and caspase activity (Figs 3D and EV4D).

Arguing for a BH3 binding site-independent interaction between E2F1 and BCL-xL, E2F1/BCL-xL co-immunoprecipitations were unaffected by WEHI-539 treatment (Fig 3A). Moreover, BRET signals between E2F1 and BCL-xL were left intact by the R139D or G138E R139L I140N substitutions in BCL-xL, which significantly affected BRET signals between BAK and BCL-xL (Fig EV4E and F). Notably, recombinant E2F1 had no detectable effect when added to BAK-expressing isolated mitochondria, it did not enhance tBID-induced cytochrome C release, and it did not derepress BCL-xL inhibition of cytochrome C release under these conditions (J.C. Martinou, personal communications). E2F1 is thus unlikely to function as a competitive inhibitor of BCL-xL to prevent its inhibition of BAK, arguing that it indirectly interferes with BAK/BCL-xL physical and/or functional interactions, as was reported for the DNA-binding domain of p53 [26,27]. To investigate this further, we explored whether E2F1 would mitigate BCL-xL control over BAK by impacting on a dynamic process, only patent in whole cell assays. Changes in BCL-xL retrotranslocation have been suggested to impact on its anti-apoptotic function [28]. To directly investigate whether BCL-xL shuttling is critical for its ability to inhibit BAK-mediated apoptosis, we compared the ability of two variants of BCL-xL (endowed with enhanced retrotranslocation properties), BCL-xL $\Delta 2$ and BCL-xL-T_{BAX} [28], to antagonize BAK-induced cell death with that of BCL-xL. These variants more efficiently prevented BAK-induced cell death, indicating that changes in BCL-xL localization dynamics impact on its control over BAK. Apoptosis suppression by BCL-xL required its mitochondrial localization since no protection was observed with the cytosolic BCL-xL A221R variant (Fig 4A and Appendix Fig S1E) [29]. Of note, BCL-xL $\Delta 2$ and BCL-xL-T_{BAX} were not detectably more efficient against BAX-induced cell death than wild-type BCL-xL (Fig 4A). This could be due to the fact that BAX is intrinsically more mobile than BAK [12]. Alternatively, the cell death rates induced by BAX in these assays may be too low to detect improved protection when BCL-xL retrotranslocation is enhanced.

We then investigated the effects of E2F1 on the subcellular localization dynamics of BCL-xL. To this end, we used MCF-7 cells stably expressing YFP-BCL-xL and performed fluorescence recovery after photobleaching (FRAP) experiments as described in [13–15]. These cells were transiently transfected with mCherry fused to E2F1 forms (wt, OTC and E132), and we investigated YFP-BCL-xL mobility between the cytosol and mitochondria in red fluorescent cells. We controlled that YFP-fused BCL-xL interacted with wt or E132 forms under these circumstances (Fig EV5A). FRAP studies on cells expressing the negative control mCherry revealed that YFP-BCL-xL recovered to about 80% of its initial fluorescence (Fig 4B). This indicates that four of five of BCL-xL molecules are mobile and that only the 20% remainders are stably associated with the outer mitochondrial membrane. In cells overexpressing E2F1, YFP-BCL-xL only recovered to 60% of its initial fluorescence, indicating a twofold increase (20–40%) in BCL-xL molecules stably associated with the outer mitochondrial membrane compared to controls. We observed the same decrease in recovery rates for the E132 or OTC forms (Fig 4B). Similar conclusions were drawn using mouse embryonic fibroblasts stably expressing GFP-BCL-xL (Fig EV5B). These experiments support the notion that E2F1, independently

from transcription, stabilizes BCL-xL association with mitochondrial membranes, thereby limiting retrotranslocation rates required, as shown above, for full inhibition of BAK.

To the best of our knowledge, our work is the first one to describe how BCL-xL subcellular localization dynamics impact on its ability to inhibit cell death (mediated by BAK in particular) and to define one regulatory element thereof, namely E2F1. Inhibition of BCL-xL retrotranslocation (upon E2F1 accumulation or mutations in its C-terminal end) might erode its anti-apoptotic activity by precluding active shuttling of BCL-xL pro-apoptotic binding partners BAK, but also possibly BAX or BH3 activators, away from their site of oligomerization and action [13,15,28]. Alternatively, it might prevent interactions of BCL-xL with non-mitochondrial effectors, involved in its anti-apoptotic function. Interestingly, changes in BCL-xL shuttling also impact on the cell response to BH3 mimetics, since membrane localization of BCL-xL selectively influences its binding to the BH3 domains of apoptotic effectors [29]. Thus, modifications in the respective amounts of mobile versus mitochondrial-stable BCL-xL molecules are functionally relevant, and they represent a critical level of regulation of BCL-xL survival function. We show here that these changes can be modulated independently from BCL-xL BH3 binding as E2F1 impacts on BCL-xL localization dynamics while interacting with it at a site that appears different from its BH3 binding one. The reported effect of E2F1 might extend to retrotranslocation of the other inhibitor of BAK, MCL-1 [30]. Of note, another implication of our work is that differences in BCL-xL localization dynamics may be found between cancer versus normal cells due to differences in E2F1 expression and activity [31]. In all cases, it underscores that the BCL-2-regulated apoptotic network has to be considered as a dynamically evolving one, which is influenced by tumor suppressor pathways not only at the level of synthesis rates and protein complex formation but also at the level of shuttling kinetics between subcellular membranes and the cytosol.

Materials and Methods

Cell culture and reagents

Cells lines were obtained from ATCC excepted the HCT116 p21^{-/-} cell line that was kindly provided by Dr Volgenstein. Saos-2 and HCT116 p21^{-/-} were cultured in Mc Coy's 5A; MCF-7 and BT-549 in RPMI₁₆₄₀; and U251 in DMEM. MCF-7 stably overexpressing YFP-BCL-xL was obtained by transfection with pYFP-BCL-xL and selection with G418. MDA-MB-231 OMI-mCherry cells were selected with puromycin after infection with retroviruses (MOI 3) containing vector coding for human OMI sequence fused to the mCherry sequence. Cells with moderate expression of OMI-mCherry were sorted with the BD-FACS ARIA III sorter. All transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions.

Unless indicated otherwise, treatments were used at the following concentrations: 2 μ M of Wehi-539 (ApexBio), 50 μ M of etoposide (Sigma), and 5 μ M of Q-VD-OPh (R&D System).

Flow cytometry

Apoptosis analysis was evaluated by staining cells with Annexin V-APC (BD Pharmingen) or with anti-cleaved caspase-3-Alexa Fluor

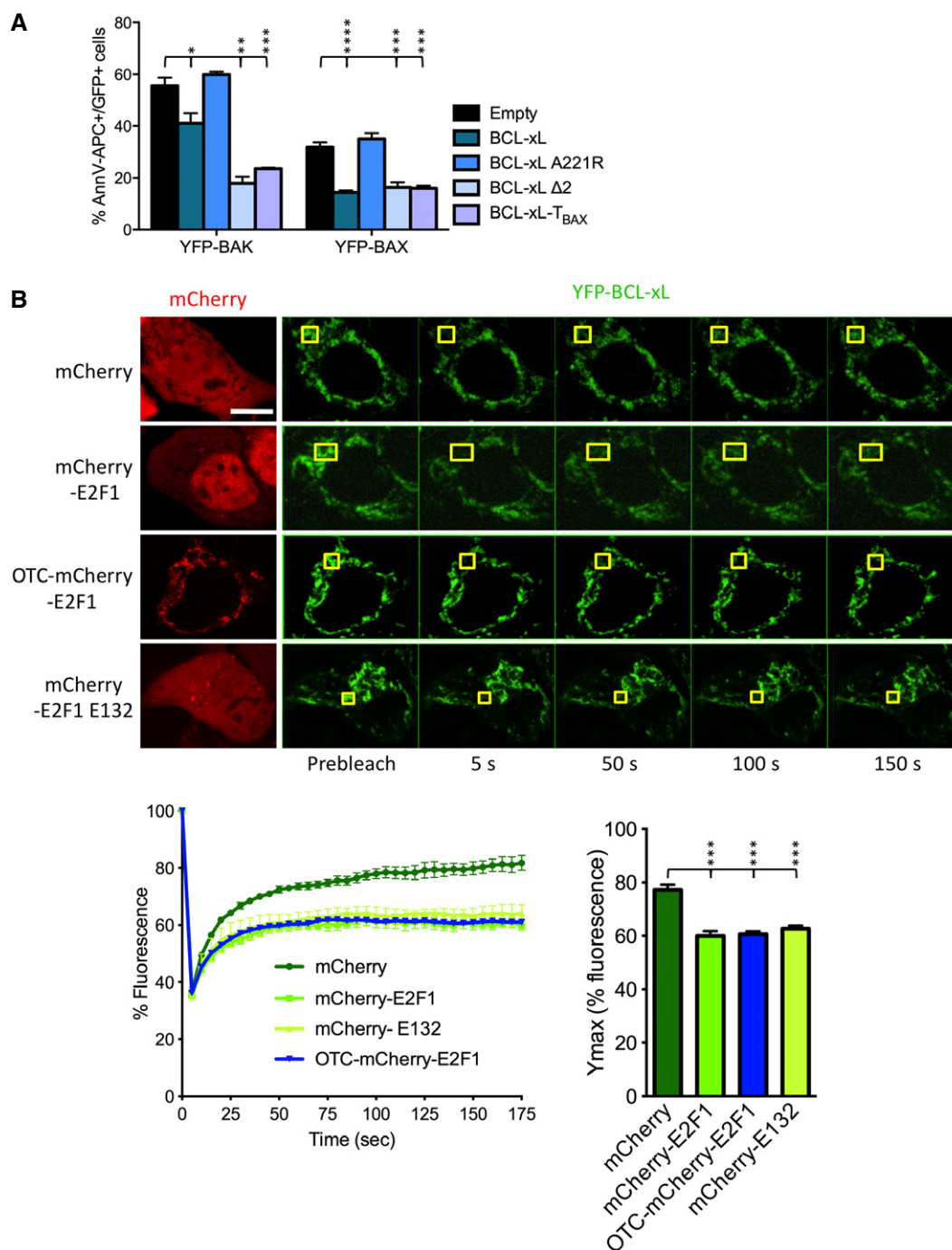


Figure 4. BCL-xL mobility is decreased by E2F1 overexpression, and it determines BAK inhibition efficiency.

A BCL-xL variants that enhanced its mobility (BCL-xL $\Delta 2$ and BCL-xL-T_{BAX}) inhibit BAK-mediated cell death more efficiently compared to BCL-xL. Saos-2 cells were co-transfected with plasmids encoding for the indicated BCL-xL variants together with YFP-BAK or YFP-BAX in a molecular ratio 3:1. 48 h later, apoptosis was analyzed as described in Fig 1D.

B E2F1 stabilizes BCL-xL on mitochondria. MCF-7 stably expressing YFP-BCL-xL transfected with the expression vectors coding for either mCherry, mCherry-E2F1, OTC-mCherry-E2F1, or mCherry-E132. 16 h post-transfection, cells were photobleached in the yellow region of interest (ROI) and imaged every 5 s (prebleaching and 5, 50, 100, 150 s after photobleaching are shown). Scale bar = 10 μ m. Fluorescence intensity was analyzed within the ROI and normalized to 100% (corresponding to fluorescence intensity before photobleaching) as shown in corresponding curves. Graph bars presented in the histogram showed the maximal percentage of fluorescence recovered after the photobleaching as determined by the fitting using a one phase exponential equation. Data presented are means of four independent experiments, corresponding to measure in at least 30 cells analyzed per condition.

Data information: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ versus control (Student's t -test). Graph bars represent the average of at least three independent experiences, and the error bars show the SEM.

647 antibody (9602, Cell Signaling) according to the manufacturer's instructions and performed on FACS Calibur (BD Biosciences) using the CellQuestPro software (Becton–Dickinson). MOMP quantification in MDA-MB231 OMI-mCherry cells was determined by evaluating the mCherry low fluorescence cell percentage using the BD-FACS ARIA III (BD Biosciences) operated by the DIVA software.

Plasmids

The pcDNA3.1, pYFP-C1, and pRLuc-C1 plasmids were used to express BCL-2 family proteins. YFP-TM_{BCL-xL} contains the 209 to 233 amino acids of BCL-xL. BCL-xL Δ 2 and BCL-xL-T_{BAK} alleles were kindly provided by F. Edlich. Human E2F1 and the Δ C, and DBD and the Δ N encoding sequences were cloned in pGFP-C1, pmCherry-C1, pRLuc-C1, or pGEX-4T1 plasmids to express the GFP, mCherry, RLuc, and GST-fusion E2F1 proteins, respectively. Δ C, DBD, and Δ N sequences lead to the expression of E2F1 amino acids residues 1–214, 114–191, and 191–437, respectively. The mitochondrial targeting sequence of ornithine carbamoyltransferase (OTC) from Lwtp53 plasmid [6] was cloned in fusion with GFP- and mCherry-E2F1 encoding sequence. L132E N133F point mutations (E132 allele) were introduced by directed mutagenesis.

siRNAs and transfection

The following siRNAs were used: siE2F1 (HSC.RNAI.N005225.10.3), siBAX (ON-TARGETplus BAX siRNA smart pool L-003308-01), siBAK (ON-TARGETplus BAK1 siRNA smart pool L-003305-00), and siControl (ON-TARGETplus Non-targeting pool D-001810-10-20). Plasmids and siRNAs were transfected according to the manufacturer's instructions (Invitrogen) using Lipofectamine 2000 and Lipofectamine RNAi Max, respectively.

RNA extraction, reverse transcription and real-time quantitative qPCR

RNAs were extracted using NucleoSpin[®] RNA (Macherey-Nagel). Reverse transcription was performed using Maxima First Strand cDNA Synthesis Kit for qPCR (Thermo Scientific). qPCR was realized on Stratagene Mx3005P thermocycler (Agilent Technologies) using Maxima SYBR Green qPCR Master Mix (2 \times) ROX kit (Thermo Scientific).

The following couple of primers were used for qPCR analysis:
 TP73: 5'- CTTCAACGAAGGACAGTCTG/AAGTTGTACAGGATGGTG GT-3'
 BBC3: 5'- ACCTCAACGCACAGTACGA/GCACCTAATTGGGCTCCAT C-3'
 BCL2L11: 5'- GCCTTCAACCACTATCTCAG/TAAGCGTTAAACTCGT CTCC-3'
 HRK: 5'- CAGGCGGAACCTGTAGGAAC/AGGACACAGGGTTTTCAC CA-3'

Immunoblot analysis and antibodies

Proteins were obtained by lysing cells with CHIP buffer (SDS 1%, EDTA 10 mM, Tris–HCl [pH 8.1] 50 mM, and proteases/phosphatases inhibitor Pierce) followed by sonication prior separation on SDS–PAGE.

The following antibodies were used: ACTIN (MAB1501R) and BIM (AB17003) from Millipore, E2F1 (3742), COXIV (4850), PUMA (4976), BID (2002S), and BAK (3814) from Cell Signaling, BAX (A3533) and BCL-2 (M0887) from Dako, BCL-xL ([E18] Ab32370) and GFP (Ab290) from Abcam, MCL-1 (sc-819), LAMIN A/C (sc-376248), and KTN (sc-33562) from Santa Cruz, and PARP (#AM30) from Calbiochem. Clarity[™] western ECL kit (Bio-Rad) was used for immunoblot revelation.

Heavy membrane fractionation and mitochondria purification

MCF7 and HeLa heavy membranes fraction were prepared by differential centrifugations as described in detail previously [32]. Saos-2 subcellular fractionation to isolate heavy membranes fraction was performed using Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific) based on differential centrifugations. Briefly, cells were lysed in the supplied buffer with proteases/phosphatases inhibitor by using a dounce homogenizer. Sequential centrifugation (3 \times 700 g 10 min and 12,000 g 20 min) leads to pellet the heavy membrane fraction. Pellet was resuspended with CHIP buffer and was used for Western blot analysis. A subcellular fraction enriched in intact mitochondria was prepared from Saos-2 cells using the MACS Technology and superparamagnetic microbeads conjugated to anti-TOM22 antibody (mitochondria isolation kit, Miltenyi Biotec). Briefly, cells were homogenized in the supplied lysis buffer by using a dounce homogenizer. Lysate was incubated with anti-TOM22 magnetic beads for 1 h at 4°C before magnetically separating the mitochondria on the MACS column. The magnetically labeled mitochondria were resuspended with CHIP buffer and were used for Western blot analysis. Total extract was obtained by directly lysing cells in CHIP buffer.

Immunoprecipitation assay

Protein lysates were obtained by lysing cells with PBS-1% CHAPS buffer containing proteases/phosphatases inhibitor and clarification at 13,000 g 15 min 4°C. Immunoprecipitation was performed on 500 μ g of protein lysates incubated with 10 μ l of anti-BCL-xL or anti-E2F1 antibodies by using the PureProteome[™] Protein G Magnetic Beads protocol (Millipore).

Pull-down assay

Recombinant proteins: GST, GST-E2F1, GST- Δ C, GST-DBD, and GST- Δ N were produced in *Escherichia coli*, prior immobilization on glutathione–sepharose (Amersham Biosciences), followed by incubation with 100 ng of recombinant BCL-xL (Biorbyt). Interactions were evaluated by immunoblotting anti-BCL-xL ([E18] Ab32370) or anti-GST (Rockland).

BRET saturation curve assays

BRET experiments were performed as described in Ref. [29]. Briefly, cells were plated in 12-well plates and transfected with increasing amounts (50–1,500 ng/well) of plasmids coding for a BRET acceptor (YFP-BCL-xL, YFP-BCL-xL R139D, YFP-BCL-xL G138E R139L I140N, YFP-TM_{BCL-xL}, or YFP-BAK), and constant amounts (50 ng/well) of plasmid expressing a BRET donor (RLuc-E2F1, RLuc- Δ C, RLuc- Δ N, RLuc-BAK, and RLuc-BCL-xL). BRET measurement was performed

using the lumino/fluorometer Mithras LB 940 (Berthold Technologies, France) after addition of coelenterazine H substrate (Interchim) (5 μ M). BRET signal corresponds to the emission signal values (530 nm) divided by the emission signal values (485 nm). The BRET ratio was calculated by subtracting the BRET signal value obtained with co-expressed donor and acceptor by that obtained with the donor protein co-expressed with untagged BCL-xL. Data shown are representative of at least three independent experiments.

Microscopy and FRAP assay

MOMP imaging was performed using HCS Array Scan Thermo. Prior fluorescence images of Saos-2, cells were incubated with 100 nM MitoTracker Red CMXRos (Life Technologies, M7512) and 1 μ g/ml Hoechst, 20 min 37°C. Acquisition was realized using Zeiss Axio Observer Z1. Live imaging was performed on a Zeiss Axio Observer Z1 with a CSU-X1 spinning disk (Yokogawa), using a 63 \times /1.40 Plan Apo lens, an Evolve EMCCD camera (Photometrics), and a motorized XYZ stage (Applied Scientific Instrumentation) driven by Marianas hardware and SlideBook 5.0 software (Intelligent Imaging Innovations). FRAP implies one region reach in BCL-xL fluorescence was bleached (one iteration, 488 nm, 100%, 10 ms), and images were captured every 5 s. FIJI software was used for analysis. Fluorescence background was subtracted, prior quantifying fluorescence of the FRAP region using ROI manager plugin in FIJI. Data were normalized to 100% fluorescence prebleaching. Statistical analysis was calculated using nonlinear regression analysis in GraphPad Prism 5.0.

Statistical analysis

Unpaired Student's *t*-test was used for statistical analysis with GraphPad Prism 5.0 Software. Errors bars represent standard errors of mean (SEM). The symbols correspond to a *P*-value inferior to *0.05, **0.01, ***0.001, and ****0.0001.

Expanded View for this article is available online.

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Author contributions

CV, SL, AF, JA, CK, LEK, and FB conducted experiments and analyzed the data. CV, SB-N, FG, LD, APG, PPJ, and LM designed the experiments and interpreted

results. PPJ and LM conceived the study, supervised it, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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