Regulation of nuclear envelope permeability in cell death and survival

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Abbreviations: NPC, nuclear pore complex; NE, nuclear envelope; Nup, nuclear pore protein; ER, endoplasmic reticulum; STS, staurosporine; TG, thapsigargin

The nuclear pore complex (NPC) mediates macromolecular exchange between nucleus and cytoplasm. It is a regulated channel whose functional properties are modulated in response to the physiological status of the cell. Identifying the factors responsible for regulating NPC activity is crucial to understand how intracellular signaling cues are integrated at the level of this channel to control nucleocytoplasmic trafficking. For proteins lacking active translocation signals the NPC acts as a molecular sieve limiting passage across the nuclear envelope (NE) to proteins with a MW below ~40 kD. Here, we investigate how this permeability barrier is altered in paradigms of cell death and cell survival, i.e., apoptosis induction via staurosporine, and enhanced viability via overexpression of Bcl-2. We monitor dynamic changes of the NPC's size-exclusion limit for passive diffusion by confocal time-lapse microscopy of cells undergoing apoptosis, and use different diffusion markers to determine how Bcl-2 expression affects steady-state NE permeability. We show that staurosporine triggers an immediate and gradual leakiness of the NE preceding the appearance of apoptotic hallmarks. Bcl-2 expression leads to a constitutive increase in NE permeability, and its localization at the NE is sufficient for the effect, evincing a functional role for Bcl-2 at the nuclear membrane. In both settings, NPC leakage correlates with reduced Ca²⁺ in internal stores, as demonstrated by fluorometric measurements of ER/NE Ca²⁺ levels. By comparing two cellular models with opposite outcome these data pinpoint ER/NE Ca²⁺ as a general and physiologically relevant regulator of the permeability barrier function of the NPC.

Introduction

In eukaryotic cells the nuclear envelope (NE) provides a physical barrier that secludes and organizes genomic material within the nucleus. Molecular traffic across the NE occurs exclusively via nuclear pore complexes (NPCs), multimeric macromolecular channels spanning both lipid layers of the NE.

The NPC acts as diffusion barrier for inert molecules with a MW larger than ~40 kD and facilitates the translocation of much larger proteins up to a flux rate of 1000/s.¹ This activity is dynamically regulated in response to physiological or pathological signaling cues.²,³ Functional control of the NPC may occur via alterations in NPC composition⁴, by post-translational modification of nuclear pore proteins (Nups),⁵,⁶ and by modulating the dimensions of the channel itself.⁷ These structural changes impinge on transport capacity and selectivity of the NPC as well as on its size-exclusion limit for passive translocation. Thus, one important layer of regulation of nucleo-cytoplasmic trafficking resides at the NPC itself⁸.

Active cell proliferation and programmed cell death are examples for cellular states known to induce modifications of NPC structure and function. Oncogene-transformed, hyperproliferating cells display larger NPC diameters than their resting counterparts.⁹,¹⁰ In mitotic cells, the level of Nup96 is downregulated to allow efficient G1/S transition.¹¹ In apoptotic cells, NPC dismantling and breakdown of the NE permeability barrier are the consequence of caspase-mediated proteolysis of a subset of nucleoporins (Nups).¹²-¹⁶ Yet another study has evidenced that calpains, Ca²⁺-activated proteases, cleave Nups in neuronal cells undergoing excitotoxic death resulting in nuclear accumulation of the cytoplasmic protein GAPDH.¹⁷ Disruption of NPC components is not the only mechanism of NE permeabilization, since nuclear leakiness has been observed also in the absence of Nup proteolysis in apoptotic and virus-infected cells.¹²,¹⁷,¹⁸ Also the proapoptotic Bcl-2 family proteins Bax and Bak were proposed to affect nucleo-cytoplasmic protein partitioning: histone H1 and nucleophosmin were shown to redistribute to the cytoplasm as a consequence of Bax/Bak overexpression independently of caspase activity.¹⁹ This result highlights yet another link between the apoptosis machinery and nucleo-cytoplasmic trafficking.

Ca²⁺ functions as second messenger in the cellular response to very diverse endogenous and exogenous signals ranging from noxious environmental insults to growth stimulatory factors.

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www.landesbioscience.com Nucleus
Numerous studies have proposed a role for Ca\(^{2+}\) in the regulation of nuclear permeability. A time course of nuclear entry of 4xCherry in HeLa cells treated with either STS (open rectangles) or TRAIL (open circles) is shown. A region of interest corresponding to the area of the nucleus was obtained by manual segmentation (see Fig. S1D). The mean intensities measured in the 4xCherry channel are plotted over time on two separate x-axes. On the left x-axis, time points from the start of image acquisition until the onset of nuclear condensation are displayed. On the right x-axis, time points immediately preceding and following nuclear condensation are shown. Here, traces were aligned with respect to the time point of nuclear condensation (time 0, dashed blue line). Red arrows indicate the time points corresponding to the frames shown in (B). Green arrows refer to the time points of Ca\(^{2+}\) measurement shown in (C) and (D). Data points are the average of at least 13 cells. Error bars show the SEM (B) Confocal images of HeLa cells expressing the nuclear permeability marker 4xCherry treated with STS or TRAIL. The time points refer to the time course shown in (A) (red arrows). (C) Characteristic traces of Fluo-4 fluorescence in HeLa cells treated to undergo apoptosis. HeLa cells were left untreated (black), or treated for 60 min with either STS (light gray) or TRAIL (dark gray), and loaded with the Ca\(^{2+}\) indicator Fluo-4. TG (5μm) was added at the indicated time point (arrow) to release Ca\(^{2+}\) from internal stores. (D) Comparison of Ca\(^{2+}\) content in internal stores after treatment with STS or TRAIL for 60 min. The difference between basal and peak fluorescence was calculated. Bars indicate the amount of Ca\(^{2+}\) mobilization in treated cells as percentage of control, untreated cells. Experiments were performed in triplicates. *** p < 0.001.

Figure 1. STS, but not TRAIL, induces a pre-apoptotic gradual increase in nuclear permeability coincident with depletion of ER/NE Ca\(^{2+}\). (A) Time course of nuclear entry of 4xCherry in HeLa cells treated with either STS (open rectangles) or TRAIL (open circles). A region of interest corresponding to the area of the nucleus was obtained by manual segmentation (see Fig. S1D). The mean intensities measured in the 4xCherry channel are plotted over time on two separate x-axes. On the left x-axis, time points from the start of image acquisition until the onset of nuclear condensation are displayed. On the right x-axis, time points immediately preceding and following nuclear condensation are shown. Here, traces were aligned with respect to the time point of nuclear condensation (time 0, dashed blue line). Red arrows indicate the time points corresponding to the frames shown in (B). Green arrows refer to the time points of Ca\(^{2+}\) measurement shown in (C) and (D). Data points are the average of at least 13 cells. Error bars show the SEM (B) Confocal images of HeLa cells expressing the nuclear permeability marker 4xCherry treated with STS or TRAIL. The time points refer to the time course shown in (A) (red arrows). (C) Characteristic traces of Fluo-4 fluorescence in HeLa cells treated to undergo apoptosis. HeLa cells were left untreated (black), or treated for 60 min with either STS (light gray) or TRAIL (dark gray), and loaded with the Ca\(^{2+}\) indicator Fluo-4. TG (5μm) was added at the indicated time point (arrow) to release Ca\(^{2+}\) from internal stores. (D) Comparison of Ca\(^{2+}\) content in internal stores after treatment with STS or TRAIL for 60 min. The difference between basal and peak fluorescence was calculated. Bars indicate the amount of Ca\(^{2+}\) mobilization in treated cells as percentage of control, untreated cells. Experiments were performed in triplicates. *** p < 0.001.

Results

Staurosporine induces a pre-apoptotic, caspase-independent leakiness of the NE. We first investigated how STS treatment affects the permeability barrier function of the NE. To this end, we performed single-cell, confocal time-lapse recordings of HeLa cells expressing a reporter for passive passage across the NE, a tetrameric mCherry fusion protein (4xCherry) (Fig. 1A and B; Fig. S1A and B). Images were taken approx. every 8 min, and a minimum of 13 cells was recorded in each experiment. The nuclear influx of 4xCherry was quantified over time as described in “Materials and Methods” and in Figure S1D. To monitor apoptosis progression the cells were stained with low concentrations of the vital DNA dye Hoechst 33342. This enabled to visualize nuclear condensation as a hallmark of caspase-dependent apoptotic execution (Fig. S1C). To account for the asynchronicity of the apoptotic process, data from each time series were split into two parts and displayed...
on separate x-axes. The left portion of the x-axis shows traces from the start of the experiment up to time points immediately preceding nuclear condensation (pre-apoptotic). On the right x-axis, traces were aligned with respect to the time of nuclear condensation (time point 0). This arrangement properly visualizes changes in nuclear permeability occurring at nuclear execution.

STS-treated cells died on average 3.5 h after drug addition, when 80% displayed apoptotic nuclei (not shown). Traces of nuclear 4xCherry fluorescence displayed a characteristic early-onset and gradual increase in signal intensity indicating that nuclei became partially accessible to the permeability marker shortly after drug addition (Fig. 1A, left x-axis, open rectangles). At later time points immediately preceding chromatin condensation, a steep increase in nuclear fluorescence was observed, suggesting a rapid collapse of the NE permeability barrier (Fig. 1A, right x-axis, open rectangles). To determine whether this behavior is generally associated with apoptotic cell demise we treated cells with TRAIL, a death receptor ligand triggering cell death via the extrinsic pathway. Cells underwent apoptosis quite rapidly as expected (~1.5h) but in contrast to STS treatment nuclei efficiently excluded 4xCherry until chromatin condensation ensued (Fig. 1A, open circles, left x-axis). In the final phase, the NE permeability barrier collapsed in these cells similarly to STS-treated cells (Fig. 1A, right x-axis). These data suggest that the observed pre-apoptotic increase in nuclear accessibility is a distinctive feature of STS-induced cell death. To address the question about a potential involvement of Ca2+ we determined ER/NE [Ca2+] levels at various time points after treatment with both drugs. We used Fluo-4 as a Ca2+-specific indicator, in combination with thapsigargin (TG), an inhibitor of the ER Ca2+-ATPase.39 TG induces the discharging of ER/NE Ca2+ resulting in an abrupt increase of its cytosolic concentration which can be detected with Fluo-4.

Thus, TG-induced Ca2+ transients are an established measure of ER/NE Ca2+ content. At 60 min after STS addition we observed a 50% reduction of ER Ca2+ levels, contrarily to cells stimulated for the same time period with the death-receptor ligand TRAIL. The latter had no significant effect on ER/NE [Ca2+] (Fig. 1C, D). These results suggest that the early increase in the size exclusion limit of the NE for passive diffusion observed under STS treatment is associated with the depletion of ER/NE [Ca2+] levels induced by this drug.

Early NE leakiness precedes apoptotic cleavage of nucleoporin Nup153 and is not dependent on caspase activity. According to previous studies, collapse of the nucleocytoplasmic permeability barrier in apoptosis is brought about by the caspase-mediated cleavage of Nups.44 To temporally correlate the permeability alterations of the NE observed in STS- and TRAIL-treated cells with NPC proteolysis we monitored a fluorescently labeled fusion of Nup153 over time. Nup153 is a component of the NPC located at the nuclear basket and is efficiently cleaved by executioner caspases.12,16 In control experiments we verified that the Nup153-GFP fusion protein is processed upon treatment of HeLa cells with STS or TRAIL in a caspase-dependent fashion (Fig. S2). In our confocal time series of apoptotic cells we then quantified the average intensity of the Nup153-GFP signal at the nuclear rim by manual segmentation, taking care to avoid regions in which the NE formed convolutions, as seen quite frequently in STS-treated cells. Such convolutions appear as out-of-focus regions of the nuclear rim signal (Fig. S1D). Both STS and TRAIL treated cells showed a clear and abrupt reduction of Nup153-GFP fluorescence at the nuclear rim at the onset of chromatin condensation. This effect was abrogated in the presence of the pan-caspase inhibitor zVAD and by inactivation of the caspase cleavage site in Nup153 at amino acid position 349 (Fig. 2, right x-axes). In the time window preceding chromatin condensation, Nup153-GFP traces were quite similar for STS and TRAIL treatment and both were not affected by zVAD (Fig. 2, left axes). According to these data, loss of Nup153-GFP signal from the nuclear rim is an indicator of caspase-dependent dismantling of the NPC which occurs concomitantly to chromatin condensation. We then investigated the effect of zVAD on the kinetics of nuclear 4xCherry influx. Caspase-inhibition prevented the final, abrupt collapse of the permeability barrier in both apoptosis models, but had no effect on the early-onset, moderate increase in nuclear accessibility observed in cells treated with STS (Fig. 3A). In these cells caspase-3-like activity was not detectable until 2h after stimulation, when partial nuclear entry of 4xCherry was already established. This assay also confirmed that zVAD was highly effective in blocking caspase activation (Fig. 3C). In sum, these results support the existence of an early, caspase-independent leakiness of the NE that precedes the apoptotic dismantling of the NPC triggered by STS.

Early pre-apoptotic permeabilization of the nuclear envelope depends on Ca2+, but not on calpain activation. The NPC has been shown to be the target of non-caspase proteolytic systems, in particular of the Ca2+-dependent protease family of calpains.

**Figure 2.** Caspase-dependent cleavage of Nup153-GFP coincides with chromatin condensation in TRAIL and STS-induced apoptosis. HeLa cells expressing Nup153-GFP (green line) or its caspase-uncleavable mutant Nup153-D349N-GFP (red line) were treated with STS or TRAIL. The former were additionally preincubated with the pan-caspase inhibitor zVAD (black line). Regions of interest corresponding to the nuclear rim were defined interactively taking care to omit convoluted regions (see also Material and Methods and Fig. S1D). Normalized Nup153-GFP signal intensities are plotted over time as in Figure 1A. The dashed blue line indicates the time point of nuclear condensation. Data points are the average of at least 15 cells. Error bars show the SEM.
In primary cortical neurons, Ca\(^{2+}\) overload caused by exposure to excitotoxic glutamate concentrations activates calpains resulting in the degradation of several Nups and the concomitant loss of proper nucleo-cytoplasmic partitioning of reporter molecules.\(^{17}\) Excitotoxic glutamate concentrations activates calpains resulting in the degradation of several Nups and the concomitant loss of proper nucleo-cytoplasmic partitioning of reporter molecules.\(^{17}\) In the degradation of several Nups and the concomitant loss of proper nucleo-cytoplasmic partitioning of reporter molecules.\(^{17}\)

To investigate the potential involvement of calpains in STS-mediated nuclear permeabilization in HeLa cells we performed live-cell microscopy experiments in the presence of the cell-permeable calpain inhibitor calpeptin. The efficacy of the inhibitor was confirmed using an in vitro calpain activity assay (Fig. S3). Differently from neuronal excitotoxicity, calpeptin did not protect from NE permeability changes induced by STS in HeLa cells both on the early and late time scale (Fig. 3B). Early nuclear leakiness, however, was efficiently prevented in the presence of BAPTA-AM, a cell permeable, high-affinity Ca\(^{2+}\) chelator that removes intracellular free [Ca\(^{2+}\)] also in internal stores.\(^{40}\) In the presence of BAPTA-AM mobilization of free [Ca\(^{2+}\)] from the ER/NE is blocked. This compound did not inhibit the collapse of the NE permeability barrier occurring at nuclear condensation (Fig. 3B). In addition, up to 2h after treatment with STS caspase-3-like activity was not significantly affected either by calpeptin or by BAPTA-AM (Fig. 3C). This lack of correlation between caspase activation and the early nuclear redistribution of 4xCherry by confocal microscopy. Passage of dextran across the NE was quantified by measuring the average fluorescence signal intensity in cell nuclei. In both HeLa and SW480 cells, Bcl-2 expression led to a marked increase in dextran nuclear entry as compared with control (Fig. 4A). This finding was confirmed with transient transfections of HeLa cells stably expressing wildtype murine Bcl-2 (HeLa-mbcl-2), and SW480 cells expressing the human counterpart (SW480-hbcl-2) (Fig. S4A) with the respective control cell lines using our Nuclear Permeability Assay (NPA)\(^{42,43}\). Cells were partially permeabilized with digitonin, incubated with fluorescently labeled 70 kD dextran, and imaged in response to the Ca\(^{2+}\) chelator further supports the existence of a caspase-independent effect of STS at the NE which requires mobilization of Ca\(^{2+}\) from internal stores. At the final stage of chromatin condensation, the NE permeability barrier collapses, a process dominated by the effect of executioner caspases and evidently not dependent either on Ca\(^{2+}\) or on calpains.

**Exogenous expression of Bcl-2 at the nuclear membrane increases NE permeability.** Bcl-2 is an antiapoptotic protein with potent pro-survival activity whose expression leads to a reduction in ER/NE Ca\(^{2+}\) levels.\(^{46}\) Bcl-2 family proteins Bax and Bak have been suggested to increase nuclear permeability\(^{19}\) as part of their proapoptotic activity. Their antiapoptotic cousin Bcl-2 has not been investigated so far. Therefore we sought to determine whether Bcl-2 expression might impinge on NE permeability and whether this would involve ER/NE Ca\(^{2+}\). First, we compared HeLa cells stably expressing wildtype murine Bcl-2 (HeLa-mbcl-2), and SW480 cells expressing the human counterpart (SW480-hbcl-2) (Fig. 4B) with the respective control cell lines using our Nuclear Permeability Assay (NPA).\(^{42,43}\) Cells were partially permeabilized with digitonin, incubated with fluorescently labeled 70 kD dextran, and imaged in response to the Ca\(^{2+}\) chelator further supports the existence of a caspase-independent effect of STS at the NE which requires mobilization of Ca\(^{2+}\) from internal stores. At the final stage of chromatin condensation, the NE permeability barrier collapses, a process dominated by the effect of executioner caspases and evidently not dependent either on Ca\(^{2+}\) or on calpains.

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we compared nuclear entry of 4xCherry in cells expressing full length Bcl-2, and Bcl-2 variants targeted either to the endoplasmic reticulum via fusion to the C-terminus of cytochrome b5 (ER-mBcl249), or to the mitochondria (mito-mBcl246) or to the NE (NE-mBcl2, this study). The latter Bcl-2 variant was generated by replacing the C-terminal transmembrane domain of Bcl-2 with the KASH domain of human Nesprin-2, a protein tethering the actin cytoskeleton to the nuclear membrane50 (Fig. S5A). At high expression levels NE-mBcl2 showed a tendency to mislocalize to the ER (not shown). We therefore expressed the corresponding gene under the control of a tetracycline-regulatable promoter in HeLa Tet Off cells. At low expression levels we could achieve an exclusive localization of NE-Bcl2 at the nuclear rim, as judged by its colocalization with Nesprin-2 in confocal images (Fig. S5B). HeLa cells were transiently transfected with plasmid vectors encoding wildtype, mitochondrial, and ER targeted Bcl-2. NE-targeted Bcl-2 was expressed in HeLa Tet Off cells in the presence of doxycyclin. NE-targeted GFP served as control. Nuclear permeability was assessed by contrasfection of 4xCherry and counting cells with equal distribution of the permeability marker between nucleus and cytoplasm. Bcl-2 localization was verified via immunocytochemistry. An increase in NE permeability comparable to that caused by wildtype Bcl-2 was obtained only in cells in which Bcl-2 localized at the ER and the NE but not at the mitochondria. We observed a 2-fold increase with respect to control cells expressing only the permeability marker (Fig. 5A, B and C). These data indicate that localization of Bcl-2 at the NE is sufficient to mediate its activity on the nuclear permeability barrier.

Bcl-2 effects at the NE are mediated by Ca²⁺. The effect of Bcl-2 as regulator of ER Ca²⁺ levels is well documented (reviewed in 48). Based on our observation in STS-treated HeLa cells suggesting that Ca²⁺ is involved in controlling the size exclusion limit of the NE, and having detected that nuclear membrane-associated Bcl-2 might increase this limit, we inferred a role for Ca²⁺ as downstream mediator of Bcl-2 at the NE. To address this question, we first determined ER/NE Ca²⁺ levels in Bcl-2-expressing cells using Fluo-4 and TG. Hela cells stably expressing wildtype Bcl-2 showed a marked reduction in TG-induced Ca²⁺ release from internal stores as compared with control cells, in line with previous studies41 (Fig. 6A and B, left panel, left bar). We also recorded capacitative Ca²⁺ influx, a secondary Ca²⁺ response ensuing in cells with depleted internal stores when Ca²⁺ is added back to the culture medium.49 In Bcl-2 overexpressing cells, this response was shown to be downregulated as adaptation to the long-term reduction of Ca²⁺ in their internal stores.50 Readdition of Ca²⁺ (1 mM) to HeLa cells previously exposed to TG evoked a steep increase in fluorophore signal indicative of Ca²⁺ capacitative influx. This effect was significantly diminished in Bcl-2 overexpressing cells (Fig. 6A and B, right panel, left bar). These data confirm that Bcl-2 overexpression leads to altered Ca²⁺ levels in the ER/NE supporting our assumption of a role of Ca²⁺ in the Bcl-2-dependent increase in NE permeability in our experimental model. We then sought to mimic the Ca²⁺-mediated, Bcl-2 dependent effect on nuclear permeability by manipulating Ca²⁺ levels in the ER/NE independently of Bcl-2. To this end, we cultured HeLa cells for 18 h in medium at low Ca²⁺ concentration (0.1 mM), a treatment leading to an adaptive reduction of Ca²⁺ levels in the ER/NE.51 These cells displayed a marked reduction of TG-induced Ca²⁺ release as well as a lowered capacitative Ca²⁺ influx as compared with controls, similarly to Bcl-2 overexpressing cells (Fig. 6A and B, both panels, right bars). Analysis of nucleo-cytoplasmic 4xCherry distribution after adaptation to low calcium conditions indicated a significant increase in the number of cells with nuclear 4xCherry, although not to the same extent as following Bcl-2 overexpression (Fig. 6C). Finally, we restored high Ca²⁺ levels in the ER/NE of Bcl-2-expressing Hela cells by coexpressing the ER Ca²⁺-ATPase SERCA2. Correction of ER/NE [Ca²⁺] by SERCA2 overexpression has been demonstrated in Bax/Bak double knockout cells, which also display a decreased [Ca²⁺]ER.52 In line with our working hypothesis, SERCA2 effectively counteracted the Bcl-2 induced increase in nuclear permeability further implicating ER/NE Ca²⁺ levels in the Bcl-2-dependent regulation of NE permeability (Fig. 7A and B).
In sum, these data show that Bcl-2 overexpression lowers the size exclusion limit of the nuclear envelope by altering Ca^{2+} homeostasis at the ER/NE, and that localization of the protein at the nuclear membrane is sufficient for the effect.

Discussion

The identification of general mechanisms that govern NPC activity in response to different extra- and intracellular signaling cues is of prime importance for understanding the NPC's recognized involvement in basic nuclear functions, not only nucleo-cytoplasmic trafficking but also chromatin organization and gene expression. The present work focuses on the NPC's activity as molecular sieve and investigates how this functional parameter is affected by a drug treatment triggering cell death, vs. a condition of increased cell survival and proliferation. In both settings we detect an increase in the size-exclusion limit of the NPC and show data indicating Ca^{2+} as a common mediator of the effect.

In STS-treated cells, we detect an early, pre-apoptotic leakiness of the NE concomitantly to a decrease in NE/ER Ca^{2+} levels. The effect is efficiently suppressed by a Ca^{2+}-chelator and mimicked by artificial downregulation of [Ca^{2+}] in internal stores. It is well established that Ca^{2+} release channels, such as IP3Rs and RyRs, carry multiple phosphorylation sites and that phosphorylation status modulates channel activity. To date at least 12 kinases are known to directly phosphorylate the IP3Rs. Treatment with STS, a pan-kinase inhibitor with broad substrate specificity, may thus directly interfere with Ca^{2+} homeostasis by altering Ca^{2+} channel activity. The association of the IP3R inhibitory protein, IRBIT, to IP3Rs is also regulated by phosphorylation. Dephosphorylated IRBIT, which might predominate in kinase-inhibited cells, cannot bind to IP3R. As a consequence, the receptor becomes sensitive to IP3 concentrations that exist in cells under resting conditions, which might result in a lower threshold for Ca^{2+} release from the NE/ER.

Ca^{2+} leakage from the ER of apoptotic cells has also been shown to result from caspase-3 dependent cleavage of IP3Rs, generating a constitutively leaky “channel-only” domain. The latter however, represents a late apoptotic event downstream of caspase activation which is difficult to reconcile with the early-stage nuclear permeabilization observed here, occurring almost immediately after exposure to STS and prior to the caspase-mediated cleavage of Nups. We can also rule out the involvement of calpain-mediated NPC proteolysis in our system, contrarily to what has been reported in neurons challenged with high-doses of glutamate to trigger excitotoxic cell death.

Our data do not support hyperphosphorylation of Nup62 as being involved in the STS-dependent early nuclear leakiness.
Nup62, a heavily O-glycosylated Nup of the central channel was shown to be excessively phosphorylated in meningo-virus infected cells displaying loss of NE permeability function. In fact, in that study, STS was used to block virus-mediated permeabilization of the NE. A more likely candidate for mediating nuclear leakiness in our experimental model is gp210, a large, N-glycosylated integral nuclear membrane protein associated with the NPC via its short C-terminus exposed toward the cytoplasm. The bulk of the protein extends into the lumen of the NE. gp210, whose cytoplasmic domain possesses five EF hand calcium-binding motives, has been proposed to function as Ca\(^{2+}\) sensor within the NE, and to mediate Ca\(^{2+}\)-dependent structural changes of the NPC. Moreover, the C-terminal cytoplasmic tail of gp210 is phosphorylated in a cell-cycle dependent manner. gp210 is required for nuclear envelope breakdown at the onset of mitosis and is possibly involved in early NPC destabilization, functions which depend upon phosphorylation of the C-terminus. Thus, gp210 could potentially respond both to alterations in NE Ca\(^{2+}\) levels and to the activity of cytosolic kinases. Whether both signaling cues converge onto gp210 and how this affects NPC structure is not known. Interestingly, stable exogenous expression of Bcl-2 in R6 rat fibroblasts which are devoid of the nucleoporin gp210 does not elicit an increase in basal nuclear permeability (data not shown) further supporting a role of gp210 in transducing Ca\(^{2+}\) signals to the NPC.

So far, the impairment of the permeability barrier function of the NPC has been mostly associated with stress conditions leading to cell demise. Thus, the finding that exogenous Bcl-2 expression, a condition leading to increased survival and stress resistance also increases basal nuclear permeability was unexpected. By showing that these cells possess a reduced steady-state luminal ER [Ca\(^{2+}\)], we provide a possible explanation to this seemingly surprising observation. We propose that both, STS treatment as well as Bcl-2 overexpression increase NE permeability via lowering luminal NE/ER [Ca\(^{2+}\)]. Bcl-2 has been shown to localize at the nuclear membrane where it could directly interact with IP3Rs. Importantly, we show that targeting Bcl-2 at the nuclear membrane is sufficient for its permeabilizing activity. Direct binding of Bcl-2 to all three IP3R isoforms has been described.

Recently these interactions have been shown to enhance the sensitivity of the IP3Rs to IP3 resulting in a decreased steady-state [Ca\(^{2+}\)] at the ER. We therefore predict that Bcl-2-IP3R interaction at the nuclear membrane will result in a similar reduction of [Ca\(^{2+}\)] in the nuclear cisterna. This correlation between ER/NE Ca\(^{2+}\) levels and NE permeability is in agreement with numerous studies on Ca\(^{2+}\)-mediated structural rearrangements of the NPC.

Our results cannot exclude the existence of further mechanisms of NE permeabilization unrelated to the NPC. High levels of Bcl-2 could destabilize the NE. Alternatively, the marked cytoskeletal deformations following STS treatment might trigger limited and controlled disruption of the NE. In parvovirus-infected cells such transient NE disruptions were reported to mediate nuclear delivery of the virus. NE deformations involving changes in the phosphorylation status of lamin A/C have been also implicated in SV-40 infection of non-dividing cells.

The observation that in STS-treated cells, early nuclear permeabilization can be prevented by BAPTA without affecting final apoptotic execution raises the question about the role of early leakiness of the NE in the apoptotic program. In the presence of BAPTA, STS-treated cells activate caspases (Fig. 3C) and undergo apoptosis. However, we could not identify nuclei with a morphology corresponding to stage 3 nuclear apoptosis (nuclear

![Figure 6](image.png)

Figure 6. Adaptation to low Ca\(^{2+}\) conditions reduces ER Ca\(^{2+}\) levels similarly to Bcl-2 expression, and mimics the Bcl-2 dependent increase in NE permeability. (A) Typical traces of Fluo-4 fluorescence in HeLa cells stably expressing Bcl-2 (light gray), and in control cells transfected with empty vector. The latter were grown either in normal medium (black) or incubated for 18h in low Ca\(^{2+}\) medium (0.1 mM, dark gray). Internal stores were depleted by TG addition (5μM, indicated by the first arrow). After recovery, extracellular Ca\(^{2+}\) was added back (1 mM, second arrow) by replacing the medium. A second phase of Ca\(^{2+}\) mobilization is observed (capacitative Ca\(^{2+}\) influx). Data points are the average of 5 cells. Error bars show the SEM. (B) Quantification of ER Ca\(^{2+}\) levels (left panel) and capacitative Ca\(^{2+}\) influx (right panel). The difference between basal and peak fluorescence values in the first and second phase of Ca\(^{2+}\) elevation are expressed as percent of the values determined in control (empty vector) cells. Experiments were performed in triplicates. * p < 0.05; ** p < 0.001. (C) HeLa control cells (empty vector) were incubated for 6h in low Ca\(^{2+}\) medium (0.1 mM) or in control medium (1mM Ca\(^{2+}\)) prior to transfection with a construct encoding the permeability marker 4xCherry. After overnight incubation, cell cultures were inspected microscopically for the presence of 4xCherry in the nucleus. Only those cells exhibiting at least a homogenous distribution of the marker between nucleus and cytoplasm were scored as positive. A total of at least 150 cells were scored. * p < 0.05.
strategies were observed upon stimulation with growth factors or during the cell cycle. In bcl-2 overexpressing cells, we observe a constitutive alteration of nuclear membrane permeability which we interpret as an adaptive response to lowered steady-state [Ca\(^{2+}\)] in the lumen of the NE. Whether this persistent enhanced accessibility of the nuclear compartment can be established only in the presence of a general death suppressor such as Bcl-2, or whether high NE permeability may promote oncogenic functions of Bcl-2 associated e.g., with cell cycle regulation or DNA repair are challenging questions for the future.

**Materials and Methods**

**Plasmid constructs.** p4xCherry was created by consecutively inserting four copies of the cDNA sequence encoding the red fluorescent GFP analog mCherry\(^{74}\) into pcDNA3.1 (Invitrogen). Removal of the internal start codons was confirmed by DNA sequence analysis. pRSET-B-mCherry was kindly provided by Roger Tsien (University of California, La Jolla) and used as template for PCR amplification.

Expression constructs encoding murine bcl-2 proteins targeted to different subcellular compartments were a kind gift of Prof. Christoph Borner (University of Freiburg): pcDNA3-mbcl2 (full length murine bcl-2), pcDNA3-mbcl2-cytb5 (ER-targeted murine bcl-2) and pcDNA3-mbcl2-RK/Chbcl2\(_{xl}\) (mitochondria-targeted murine bcl-2). The inducible expression construct for NE-targeted murine bcl-2 (NE-Bcl-2) was designed in analogy to a previously described NE-targeted GFP protein.\(^{75}\) The KASH domain of human Nesprin-2 (aa 6833–6883) was amplified from pEGFP-C1-Tm-Nesprin-2 (kind gift of Dr. Akis Karakesisoglou, University of Durham) by PCR and introduced into the EcoRI and BamHI sites of pTRE MCS (Clontech) resulting in pTRE-KASH. pTRE-KASH-mBcl-2 was obtained by inserting a mbcl2 fragment from pcDNA3-mbcl2 lacking the transmembrane domain of bcl-2 into pTRE-KASH. The control plasmid pTRE-GFP-KASH was constructed by insertion of the EGFP coding sequence into pTRE-KASH via PCR.

The pSERCA2 expression construct was kindly provided by Luca Scorrano (University of Padova, Italy). pNup153-GFP was a kind gift of Jan Ellenberg (EMBL Heidelberg). pNup153-D349N-GFP was generated by PCR mutagenesis using the following primers: 153D349NFwd 5'-GTG GGA TAG ATA TCA CAA ATT TTC AGG CCA AAA GAG AAA AG-3' and 153D349N-Rev 5'-CTT TTC TCT TCT TCT TCT TGT CCT GTG AAA ATT TGT GAT ATC TAT CCC AC-3'.

**Cell culture and transfections.** HeLa cervix carcinoma cells stably expressing murine bcl-2 (HeLa-mbcl-2) and SW480 colon carcinoma cells stably expressing human Bcl-2 (SW480-bcl-2) and the respective empty vector control cell lines were a kind gift of Prof. Christoph Borner (University of Freiburg).

HeLa Tet Off cells expressing the tetracycline controlled transactivator (Clontech) were kindly provided by Dr. Thomas Meergans (University of Konstanz).

All cell lines were maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum.
(Invitrogen) and 10% fetal calf serum (Sigma). Cells were cultivated at 37°C with 5% CO₂ in a humidified atmosphere. For live cell microscopy, cells were seeded in glass-bottom dishes (ibidi) and transfected with Effectene (Qiagen) according to the manufacturer’s instructions 24 h prior to the experiment.

HeLa Tet Off cells were transfected using the calcium phosphate co-precipitation method. Cells were used for further experiments ~24 h after transfection.

For bead loading experiments, cells were seeded in MatTek glass bottom culture dishes with a glass inset of -1 cm² and grown to a density of ~60% prior to the experiment.

Live cell imaging. Imaging experiments were performed in culture medium devoid of Phenol Red to reduce background fluorescence. After exchanging the medium, 150 µg/ml Hoechst 33342 was added for labeling nuclear DNA, and the cells were incubated at the microscope stage at 37°C and 5% CO₂ for 1–2 h before starting image acquisition. Confocal time-lapse series were recorded at a Zeiss LSM510 Meta confocal microscope equipped with a stage-top incubator and an objective heater using a 63x/1.4 NA Plan-Apochromat objective lens (Carl Zeiss Microimaging). Laser settings were optimized to ensure cell viability for the whole duration of the experiments. As an additional control for phototoxicity, it was verified that imaging conditions were compatible with cell division (Fig. S6A). Cell nuclei were tracked and focused using an autofocus macro kindly provided by J. Ellenberg. For each experiment, 10–13 cells were imaged at a 5x zoom in a time interval of about eight minutes. If necessary, a delay between acquisition rounds was introduced to keep this time frame constant. At each position, a z-stack consisting of three sections at a spacing of 0.6 µm was recorded. After the first imaging cycle, the acquisition routine was paused and the apoptotic inducer STS (Sigma Aldrich, 0.5 µM) or IZ-TRAIL (kind gift of H. Walczak, 300 ng/ml) was added. Inhibitors were added 30 min prior to STS addition at the following concentrations: zVAD-fmk (20 µM), calpeptin (5 µM) and BAPTA-AM (10 µM). For bleaching correction, time courses of untreated cells were recorded under identical conditions.

Quantification of confocal time series. Image analysis was performed using the open source software Image J. Image series were visually inspected and for each time point one z-section showing the nuclear rim in focus in the Nup153-GFP channel was selected. The images were combined into new stacks for quantification. This procedure was necessary because cells alter their morphology during the apoptotic process.

For the same reason, automatic segmentation of the cell nucleus in the Hoechst channel was not applicable. Therefore, all three channels were segmented interactively. Nuclear condensation and NE permeabilization were measured in the Hoechst and the 4xCherry channel, respectively. To this end, a ROI corresponding to the inner boundary of the nucleus was first defined in the Nup153-GFP channel and copied into the Hoechst and the 4xCherry images. For quantifying NE integrity, signal intensities were measured in the Nup153-GFP channel in a second ROI including the nuclear rim (Fig. S1D). Folded regions of the NE were omitted. Intensity values were background subtracted, corrected for bleaching, and normalized. The time point of nuclear condensation in each time series was defined as an increase in Hoechst fluorescence by a factor of at least 1.07 occurring within two consecutive frames. Cells go through apoptosis asynchronously. To better visualize the abrupt changes in nuclear permeability and NE integrity occurring at the final stage of nuclear execution, intensity values for these two channels were plotted on two separate time-axis. In the first part of the time course intensity values were displayed from the start of image acquisition up to the time point immediately preceding nuclear condensation. In the second part, traces were aligned with respect to the time point of chromatin condensation as determined in the Hoechst channel.

Assessment of nuclear permeability. Nuclear Permeability Assay (NPA) was performed as described previously. Briefly, cells were seeded onto glass coverslips ~20 h before the start of the experiment. Cell membranes were selectively permeabilized with digitonin (24 µg/ml). The semi-permeabilized cells were incubated with the fluorescent permeability marker 70 kDa Texas Red-labeled dextran (Sigma) at a concentration of 20 mg/ml and then imaged at the confocal microscope (Zeiss LSM510 Meta). Images were quantitated semi-automatically by a custom-made software extracting the average nuclear fluorescence intensity. Fluorescence intensity in the nucleus is a measure of nuclear penetration of 70 kDa dextran and thus an indicator of passive permeability of the nuclear envelope.

For the experiment shown in Figure S3B cells were loaded with 70 kDa Texas Red-labeled dextran using glass beads. Briefly, glass beads with a diameter of ~100 µm (Sigma) were treated overnight with 5 M NaOH, washed with ethanol and dried by evaporation. The culture medium was removed and 20 µl dextran solution was added to the glass inset of MatTek culture dishes (20 mg/ml 70 kDa Texas Red-labeled dextran (Sigma) in PBS). Subsequently, a monolayer of clean glass beads was added into the dish cavity and the dish was quickly shaken. Beads were then removed by washing with PBS, medium added back and the cells incubated for 45 min at 37°C. Confocal images were taken and evaluated as described above.

In cells expressing Bcl-2 and its mutants, nuclear permeability was assessed by measuring the percentage of cells displaying nuclear localization of 4xCherry. Images were taken at fixed time points after transfection of 4xCherry and Bcl-2 expression constructs by confocal fluorescence microscopy. Only cells showing at least the same 4xCherry signal in the nucleus as in the cytosol were scored as having a nuclear 4xCherry localization.

Immunocytochemistry. Immunocytochemical detection of mbc1 was performed using antibodies specific for murine bcl-2 (mouse monoclonal anti-bcl-2 clone 10C4, Santa Cruz), at a dilution of 1:500 in 10% NGS/PBS. Cells were shortly washed with 5 mM MgCl₂/PBS, permeabilised with 0.3% digitonin/PBS for 5 min on ice and rinsed again in 5 mM MgCl₂/PBS. Fixation was performed with 4% PFA/PBS for 10 min at room temperature followed by washing in PBS. Reactive aldehyde groups were blocked with 50 mM NH₄Cl for 10 min. Unspecific binding sites were saturated with 1% BSA/PBS for 30 min. Incubation with the 10C4 anti-bcl-2 antibody was overnight at 4°C. The secondary antibody conjugated to Alexa488 (Molecular Probes) was...
diluted 1:400 in 10% NGS/PBS and incubated for 1 h. Excessive washing steps between incubations removed unbound antibodies. DNA was stained with 200 ng/ml Hoechst 33342 for 10 min.

For co-detection of 4xCherry and Bcl-2, cells were fixed overnight at 4°C in 4% PFA/PBS to prevent loss of the permeability marker during permeabilization. After fixation cells were permeabilized for 5 min with 0.1% Triton X-100/PBS at room temperature, rinsed in PBS and incubated in 50 mM NH4Cl/PBS for 10 min. Subsequently, cells were treated with cold acetone at -20°C for 8 min, rinsed in PBS and incubated in 1% BSA/PBS for 30 min. Antibody incubations and Hoechst staining of DNA were performed as described above.

Immunostaining of Bcl-2 at the nuclear membrane required a permeabilization step prior to fixation and was therefore not compatible with simultaneous detection of 4xCherry. In this case, the protocol used for co-detection of 4xCherry and Bcl-2 resulted in a punctuate staining pattern that nevertheless allowed for the identification of cotransfected cells and image quantification. The correct localization of NE-Bcl-2 was confirmed in parallel transfections using digitonin permeabilization.

For co-detection of 4xCherry and SERCA2, cells were fixed overnight at 4°C in 4% PFA/PBS and processed as described above with omission of the acetone treatment. The SERCA2 antibody (IID8, Alexis) was diluted 1:750 in 1% BSA/PBS.

Preparation of whole cell extracts and immunoblot analysis. For the preparation of cell lysates, HeLa cell cultures were placed on ice, and protease inhibitors (Complete Mix; Roche Applied Science) and dithiothreitol (1mM) were added directly to the growth media. The cells were then gently scraped off the dish with a rubber policeman, washed in ice-cold PBS, resuspended in 95°C lysis buffer (50 mM Tris/HCl, pH 8.0, 0.5% SDS, 1 mM dithiothreitol), and heated at 95°C for 10 min. The cell debris was removed by centrifugation at 20,000 x g for 10 min. For the detection of Nup-153, SDS-PAGE was performed according to Thomas and Kornberg, while for other proteins it was according to Laemmli. Proteins were blotted onto nitrocellulose using a wet blot chamber (Bio-Rad Trans-Blot Cell) or a Semidry Blotter (Biometra), and filters incubated in TNT with milk overnight. After fixation cells were permeabilized for 5 min with 0.1% Triton X-100/PBS at room temperature, rinsed in PBS and incubated in 50 mM NH4Cl/PBS for 10 min. Subsequently, cells were treated with cold acetone at -20°C for 8 min, rinsed in PBS and incubated in 1% BSA/PBS for 30 min. Antibody incubations and Hoechst staining of DNA were performed as described above.

Fluorometric determination of protease activity, and of ER/NE [Ca2+]. For measurement of caspase activity, a total of 3 x 10^5 HeLa cells were seeded in six-well plates 24 h prior to the experiment and treated with STS (0.5 μM). To block caspase activation zVAD-fmk (20 μM) was added 30 min prior apoptotic stimulation. At the indicated time points, the cells were placed on ice and after the addition of protease inhibitors, they were gently scraped off the dish and collected by centrifugation. Cells were lysed in 25 mM HEPES, pH 7.5, 5 mM MgCl2, 1 mM EGTA, and 0.5% Triton X-100, and the cleavage of DEVD–7-amino-4-trifluoromethyl coumarin (afc) (40 μM) was monitored fluorometrically in reaction buffer (50 mM HEPES, pH 7.5, 10 mM dithiothreitol, 1% sucrose, 0.1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate) over a period of 20 min at 37°C, with a λ_em (emission wavelength) value of 390 nm and a λ_ex (excitation wavelength) value of 505 nm. The activity was calibrated by using afc standard solutions. Measurements were run in triplicate.

Calpain activity was measured using a Calpain Activity Assay Kit (Abcam, ab65308) according to the manufacturer’s instructions. For apoptosis induction, HeLa cells were treated with 0.5 μM STS for 2 h. To block calpain activity, 5 μM calpeptin (Merck) was added 30 min prior to STS treatment. Cell lysates were prepared from 2 x 10^6 cells. Protein concentrations were determined using BCA (Pierce Biotechnology). Lysates were spiked with 10 μM μl active calpain. After addition of the calpain substrate (Ac-LLY-afc), samples were transferred to a black UV-light permeable 96-well plate and incubated at 37°C for 60 min. Fluorescence emission of afc was detected as above.

For Ca2+ measurements, cells were cultured in 96 multiwell plates for 24 h and washed carefully with Ca2+-buffer (140 mM NaCl, 5 mM KCl, 1mM CaCl2, 1 mM NaH2PO4, 1mM MgSO4, 5.5 mM Glucose, 20 mM Hepes pH 7.4). Cells were loaded with the [Ca2+] indicator Fluo-4 (Invitrogen) by incubation in 150 μl loading buffer (Ca2+-buffer containing Fluo-4 and pluronic acid at final concentrations of 4 μM and 0.08% respectively) for 60 min at RT in the dark. After two washings with Ca2+-buffer containing 1mM probenecid, cells were incubated for 30 min at RT for complete de-esterification of intracellular Fluo-4. Immediately before measurement, the buffer was replaced with fresh Ca2+-buffer with probenecid. Fluorescence emission was detected with a microplate fluorescence reader (Genios Plus, Tecan) equipped with appropriate filters (λ_ex = 488 nM, λ_em = 510–570 nM). After measurement of basal fluorescence, 50 μl of buffer solution were replaced with 50 μl of thapsigargin (TG) containing Ca2+-buffer (final TG concentration 5 μM). TG induces release of Ca2+ from internal stores. After Fluo-4 signal intensities had recovered to the basal level (~10 min) the medium was replaced with Ca2+-buffer (1 mM Ca2+) to measure the capacitative Ca2+ influx. To measure ER/NE Ca2+ and capacitative Ca2+ influx in cells adapted to low Ca2+ conditions, cells were seeded in 96 multiwell plates two days prior to the experiment. After 24 h the growth medium was exchanged with low-Ca2+-buffer (Ca2+-buffer containing 0.1 mM Ca2+). The cells were adapted to low Ca2+ conditions for 24 h. Fluorometric measurements were performed as described above using low-Ca2+-buffer for loading the cells with Fluo-4 and subsequent washing steps. Capacitative Ca2+ influx
was measured after readdition of Ca\textsuperscript{2+}. Control measurements using Ca\textsuperscript{2+}-buffer (1 mM) were conducted in parallel.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here:
http://www.landesbioscience.com/journals/nucleus/article/21982/
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