A Critical Re-evaluation of "*Inhibition of oligomeric BAX by an anti-apoptotic dimer*" by Newman *et al*., *Cell* 2025; doi:10.1016/j.cell.2025.10.037

Mengxi Zhu, Ruihuang Wang, Yujie Zhu and Shu-Feng Zhou*
College of Chemical Engineering, Huaqiao University, Xiamen 361021, China
*Correspondence: szhou@hqu.edu.cn

Abstract

Newman *et al*. (*Cell*, 2025) propose a provocative revision to the canonical apoptotic regulatory model by suggesting that an anti-apoptotic BCL-2 family dimer can directly inhibit pre-formed oligomeric BAX, thereby suppressing mitochondrial outer membrane permeabilization (MOMP) after BAX activation has already commenced. This commentary critically evaluates the conceptual framework, methodological rigor, and empirical robustness underlying this claim. While the study introduces an intriguing possibility that apoptosis can be modulated at a post-oligomerization stage, several aspects of the experimental design do not fully support the strength of the mechanistic conclusions. Cryo-EM density maps lack the resolution necessary to unambiguously define the proposed inhibitory interface. Biochemical assays insufficiently document protein purity, oligomeric state, and stoichiometry, leaving open alternative interpretations regarding indirect or non-specific interactions. Cellular assays rely heavily on overexpression systems that may artificially stabilize interactions not observed under physiological conditions. Across multiple figures, critical controls are absent, including validation of BAX activation states, quantitative comparison of oligomer sizes, and longitudinal analyses of MOMP kinetics. Extended Data and Supplementary materials reveal inconsistencies in reporting, incomplete annotation of experimental conditions, and missing raw datasets essential for reproducibility. Collectively, while the hypothesis of an anti-apoptotic dimer acting directly on active BAX is compelling and potentially transformative, the evidence provided by Newman *et al*. is insufficiently rigorous to substantiate a paradigm shift. Further structural, biochemical, and live-cell mechanistic studies are required to determine whether the proposed mechanism reflects a universal mode of apoptotic regulation or an artefactual consequence of the experimental systems employed.

1. Introduction to BAX Biology and the Anti-Apoptotic Paradigm

1.1. Historical Evolution of BAX as A Central Effector of Mitochondrial Apoptosis

The Bcl-2 family of proteins plays a crucial role in regulating apoptosis, or programmed cell death[1]. This family is composed of proteins that are classified into three main groups based on their structure and function. The anti-apoptotic proteins, including Bcl-2, Bcl-XL, and MCL-1, prevent apoptosis by inhibiting the activation of pro-apoptotic proteins. These proteins contain at least four BCL-2 homology (BH) domains, which enable them to bind and sequester BH3-only pro-apoptotic proteins, thereby maintaining mitochondrial integrity and preventing mitochondrial outer membrane permeabilization (MOMP)[1]. The pro-apoptotic effector proteins, such as BAX and BAK, promote apoptosis by oligomerizing and forming pores in the mitochondrial membrane in response to cellular stress. These proteins contain a conserved BH3 domain and are responsible for inducing MOMP, which leads to the release of pro-apoptotic factors like cytochrome *c*. The BH3-only proteins, such as BIM, tBID, and PUMA, act as sensors of cellular stress and initiate apoptosis by either binding to and neutralizing anti-apoptotic proteins or by activating pro-apoptotic effectors. These proteins contain only the BH3 domain and play a key role in the initiation of the apoptotic cascade. The balance between pro- and anti-apoptotic Bcl-2 family proteins determines cell fate, and dysregulation of this balance is associated with various diseases, including cancer, neurodegeneration, and autoimmune disorders.

BAX has long been understood as a pivotal executioner within the intrinsic apoptosis pathway, functioning as one of the primary mediators of mitochondrial outer membrane permeabilization[2,3]. Since its discovery in the 1990s, BAX has been positioned within the BCL-2 family hierarchy as a latent cytosolic protein that undergoes a series of conformational transitions before integrating into the mitochondrial membrane[4]. Classical studies established that apoptotic stimuli induce BAX translocation to mitochondria, exposure of its BH3 and C-terminal helix, and multimerization into higher-order oligomers that puncture the membrane and permit cytochrome c and other apoptogenic factors to escape into the cytosol. This sequence of events has been regarded as largely irreversible once oligomerization has occurred, marking the commitment point beyond which the cell is destined to die. Early conceptual frameworks therefore positioned BAX oligomers as terminal structures resistant to inhibition by upstream anti-apoptotic molecules.

As structural biology techniques advanced, particularly NMR and crystallography applied to various BAX fragments, the field refined its understanding of activation

steps, including BH3-only protein engagement, helix α9 exposure, and transmembrane insertion. Nevertheless, the dogma persisted that once BAX forms membrane-embedded oligomers, anti-apoptotic proteins can no longer restrain its activity. This made intuitive sense because anti-apoptotic BCL-2 proteins were generally thought to act either by sequestering BH3-only activators or directly engaging latent BAX to prevent its activation. Newman *et al*.[5] challenge this long-standing assumption by suggesting that an anti-apoptotic dimer directly engages active, oligomeric BAX and reverses its death-inducing activity. The implications are far-reaching because they propose a level of regulatory plasticity at a stage previously believed to be mechanistically locked.

1.2. Structural Transitions Governing BAX Activation and Oligomerization

The activation of BAX is a complex and finely tuned process involving multiple conformational checkpoints that historically have been dissected using biochemical reconstitution, mutational studies, and limited structural snapshots[2]. In its inactive state, BAX adopts a globular fold stabilized by intramolecular packing of its helices, particularly helix α9, which tucks into the hydrophobic groove. Upon interaction with BH3-only proteins such as tBID or BIM, BAX undergoes a dramatic conformational rearrangement. This rearrangement includes the displacement of α9, exposure of the BH3 domain, and subsequent membrane recruitment.

Once docked at the mitochondrial outer membrane, BAX undergoes symmetric homodimerization through BH3-in-groove interactions[2]. These dimers then nucleate the assembly of higher-order oligomers, with additional interfaces forming between helices α2–α5 and other regions per current models. Cryo-electron tomography and biochemical crosslinking have identified ring-like or arc-like oligomeric arrangements believed to mediate pore formation. These pores enable the release of soluble proteins that initiate downstream caspase cascades.

Critical for the current study is the presumption, based on decades of research, that these oligomers are stable and resistant to disassembly by regulatory factors after formation. The hypothesis advanced by Newman *et al*.[5], that an anti-apoptotic dimer engages oligomeric BAX to inhibit or disassemble these structures, challenges foundational structural assumptions. It implies that oligomers may contain dynamic or reversible elements not accounted for in existing models. Such a hypothesis raises the bar for structural and biochemical evidence since it departs from the widely accepted understanding of BAX as a commitment-stage effector.

1.3. Anti-Apoptotic BCL-2 Family Members and Their Classical Regulatory Modes

Anti-apoptotic proteins such as BCL-2, BCL-XL, and MCL-1 have been extensively characterized as guardians of mitochondrial integrity that act upstream of BAX activation[1]. Their canonical roles fall into two main categories: sequestration of BH3-only activators and direct inhibition of latent BAX or BAK by engaging their hydrophobic grooves. The hydrophobic groove of anti-apoptotic proteins typically accommodates the BH3 domain of pro-apoptotic proteins, forming a tight complex that prevents BAX from transitioning into its activated conformation. This regulatory mechanism has been supported by structural studies, co-crystal complexes, thermodynamic analyses, and genetic screens.

Importantly, anti-apoptotic proteins have not been thought to interact significantly with active or oligomerized BAX. Instead, models depict anti-apoptotic family members acting early, before the activation threshold is crossed. If BAX becomes fully activated and oligomerized, anti-apoptotic proteins were believed to be largely bypassed. This hierarchical arrangement has guided therapeutic strategies, including BH3 mimetics that competitively inhibit anti-apoptotic proteins to free pro-apoptotic factors. These strategies assume that once BAX and BAK oligomerize, the cell is irrevocably committed to apoptosis.

By proposing that an anti-apoptotic dimer inhibits BAX after oligomerization, Newman *et al*.[5] place anti-apoptotic proteins at a later decision point in the apoptotic timeline. This interpretation would require revisiting how anti-apoptotic proteins are classified, how BH3 mimetics function, and how we interpret dose–response relationships in apoptosis assays. Any such shift necessitates a high level of evidentiary rigor.

1.4. Conceptual Shift Introduced by Newman *et al*.: Direct Targeting of Oligomeric BAX

Newman *et al*.[5] attempt to redefine the mechanistic landscape of apoptosis by suggesting that anti-apoptotic regulatory input persists even after BAX has formed oligomeric structures. According to their model, an anti-apoptotic dimer physically associates with active BAX oligomers, inhibiting membrane permeabilization. If correct, this represents a paradigm shift in how mitochondrial apoptosis is understood, implying that oligomers are far more dynamic and regulatable than previously believed.

However, the claim introduces substantial mechanistic tension with established models. It challenges the idea that BAX oligomerization represents a biochemical checkpoint that is difficult to reverse. It also raises questions about how the

proposed dimer structurally accesses oligomer interfaces embedded within the membrane environment. Any model that introduces a new regulatory layer at such a late apoptotic stage must therefore be supported by detailed structural, biochemical, and cellular evidence. As will be demonstrated in later sections, the evidence provided by Newman *et al*. is intriguing yet insufficiently robust to conclusively support the magnitude of the proposed conceptual shift.

2. Summary of Newman *et al*.'s Central Claims

2.1. Mechanistic Proposition of An Anti-Apoptotic Dimer That Inhibits BAX Oligomers

Newman *et al*.[5] advance a bold mechanistic assertion that fundamentally challenges prevailing BAX biology: they propose that a specific anti-apoptotic BCL-2 family dimer directly inhibits active, oligomeric BAX after membrane insertion has already occurred. According to their model, this anti-apoptotic dimer recognizes and binds a particular surface exposed in oligomerized BAX, leading to partial or complete suppression of mitochondrial outer membrane permeabilization. This interaction purportedly interferes with the oligomer's ability to expand or maintain pore-like structures required for cytochrome c release. By doing so, the study suggests that the dimer acts at a downstream regulatory checkpoint rather than at the classical upstream points of BH3-only sequestration or latent BAX inhibition. This central claim forms the framework for all subsequent biochemical, structural, and cellular experiments presented in the paper. If correct, the mechanism would extend anti-apoptotic regulation to later stages of apoptosis, thereby reframing how commitment to cell death is conceptualized at the molecular level.

The authors argue that this dimer possesses a unique conformational arrangement that differentiates its activity from monomeric anti-apoptotic proteins. They imply that dimerization creates a composite binding surface capable of engaging active BAX in a manner inaccessible to either monomer alone. This hypothesis, however, requires a high-resolution structural rationale, including clear delineation of interacting interfaces and binding stoichiometry, yet such evidence remains incomplete or ambiguous across the figures. Nevertheless, the central mechanistic proposition is that the anti-apoptotic dimer counteracts BAX function at a stage previously deemed irreversible.

2.2. Structural Interfaces Reported to Mediate Dimer–BAX Interactions

The structural claims of Newman *et al*.[5] hinge on cryo-EM and modeling-based identification of putative contact interfaces between the anti-apoptotic dimer and oligomeric BAX. The authors depict a surface on the dimer that purportedly

recognizes a region spanning helices α4 through α6 on activated BAX. These helices have been implicated historically in oligomer stabilization and membrane insertion, making the region an attractive target for inhibitory engagement. The paper asserts that the dimer clamps onto this structural domain in a manner that interferes with oligomer expansion or pore maturation. According to the authors, the resulting complex stabilizes a partially inactive form of BAX that is believed to be incapable of forming functional membrane pores.

However, the structural evidence supporting these interface assignments is limited by the resolution of the cryo-EM maps and the interpretive assumptions used during model fitting. Several densities are weak, nebulous, or discontinuous, requiring flexible fitting rather than unambiguous structural assignment. In many maps, the boundaries of the dimer are indistinct, raising questions about the legitimacy of the proposed interface. Furthermore, oligomeric BAX assemblies are notoriously heterogeneous and difficult to resolve, complicating the claim that specific interface geometries can be definitively deduced. The absence of orthogonal validation, such as site-specific crosslinking or mutational rescue experiments with rigorous functional quantitation, restricts confidence in the proposed interface model. Consequently, although the authors describe a plausible interaction surface, the structural underpinning of the dimer–BAX engagement remains speculative.

2.3. Proposed Biological Consequences on MOMP, Cytochrome *c* Release, and Cell Fate

The biological significance of this proposed inhibitory mechanism is framed around its capacity to modulate MOMP and thereby fine-tune cell fate decisions. Newman *et al*.[5] argue that the dimer reduces cytochrome c release by preventing full expansion of BAX pores. Several live-cell imaging experiments and biochemical analyses are presented as supporting evidence, including time-lapse microscopy of mitochondrial permeabilization, immunoblot assays tracking cytosolic cytochrome *c*, and caspase activation measurements. These data are used to support the notion that BAX pore formation remains regulatable even after oligomerization.

In theory, such a mechanism would introduce a new layer of apoptotic control at a critical juncture in mitochondrial dysfunction. The authors suggest that this post-oligomerization regulation could help cells survive transient apoptotic stimuli or modulate sensitivity thresholds in tissues where apoptosis must be tightly controlled. They further imply that the dimer's activity may serve as a protective mechanism during stress responses, mitigating accidental or excessive apoptosis.

However, the biological data provided in the study suffer from several limitations. Many experiments rely heavily on overexpression of both the dimer and BAX, raising the possibility that non-physiological protein levels drive interactions not

representative of endogenous systems. Quantitative analyses of MOMP dynamics are limited, with insufficient time resolution and incomplete normalization across biological replicates. Furthermore, the downstream consequences on caspase activation are not systematically linked to the structural or biochemical data, weakening the explanatory coherence of the study. As a result, although the proposed biological consequences are conceptually consistent with the mechanistic model, the supporting evidence remains inconclusive.

2.4. Theoretical Implications for Apoptotic Threshold Modulation across Cell Types

By suggesting that BAX oligomers remain regulatable after activation, Newman *et al*. introduce a theoretical framework where apoptotic thresholds become tunable at later stages than previously recognized. This idea diverges from classical perspectives that treat MOMP as a point of no return. If the model is correct, cells may possess a physiologically relevant mechanism for reversing or dampening MOMP even after BAX oligomers form. Such an insight would have profound implications for tissues with high apoptotic sensitivity, such as neurons, cardiomyocytes, and hematopoietic lineages.

The study further implies that variability in anti-apoptotic dimer expression could explain differential apoptotic susceptibility across cell types. This concept aligns with broader theories of apoptosis threshold tuning but conflicts with established observations that once cytochrome *c* is released, apoptosis proceeds rapidly and irreversibly. For the model to gain widespread acceptance, robust evidence must show that BAX pore formation itself is not an all-or-nothing event but is instead subject to reversible regulation.

Newman *et al*.[5] gesture toward such a model but do not supply rigorous experimental validation across diverse cell types or physiological conditions. The commentary sections that follow will discuss how methodological gaps, incomplete datasets, and interpretive inconsistencies weaken the strength of these theoretical implications. Nonetheless, Section 2 captures the essence of the study's central claims and underscores why verification of these claims would require substantially stronger evidence than what is currently presented.

3. Experimental Framework and Methodological Appraisal

3.1. *In Vitro* Biochemical Reconstitution and Protein Purification

The biochemical foundation of the Newman *et al*. study[5] rests on *in vitro* reconstitution assays intended to demonstrate direct interactions between the anti-apoptotic dimer and oligomeric BAX. These assays include recombinant protein

production, detergent-based oligomerization of BAX, liposome permeabilization systems, and co-immunoprecipitation experiments designed to detect binding events. While such approaches are standard in apoptosis research, their interpretive power relies heavily on rigorous documentation of protein purity, conformational state, and stoichiometry. In the present study, several methodological weaknesses undermine confidence in the biochemical results.

The most significant concern lies in the purification workflow. The authors provide only partial chromatographic traces and SDS–PAGE gels, without mass spectrometric validation or functional assays confirming that the recombinant dimer adopts the intended conformation. The absence of clear monodispersity profiles and incomplete reporting of buffer compositions makes it difficult to determine whether the proteins used in binding assays accurately reflect native physiological states. BAX oligomerization, in particular, is notoriously sensitive to buffer conditions, detergent choice, and lipid composition. The study does not provide an adequate comparison between detergent-induced oligomers and membrane-inserted oligomers, which limits interpretability of downstream binding experiments. Furthermore, the lack of negative controls using mutant BAX variants that cannot oligomerize restricts the ability to distinguish specific interaction events from nonspecific protein clustering.

Overall, the *in vitro* biochemistry presented by Newman *et al*.[5] suggests a possible dimer–BAX interaction but falls short of establishing the rigor needed for definitive mechanistic interpretation. The study would have benefited from a more explicit biophysical characterization of oligomer species and their relevance to the physiological membrane environment.

3.2. Cryo-EM and Structural Modelling Used to Define the Dimer Complex

Cryo-electron microscopy serves as the structural backbone of the paper's central claim that the anti-apoptotic dimer physically engages oligomeric BAX in a specific, functionally relevant manner. The authors present several density maps purported to depict the assembled complex, along with atomic models fitted into those densities. However, significant methodological uncertainties weaken the structural conclusions.

The reported resolution of the maps falls short of the threshold needed for confident side-chain assignment or precise delineation of protein–protein interfaces. Many of the densities attributed to the dimer are diffuse or inconsistent across classes, suggesting either sample heterogeneity or incomplete particle alignment. The authors rely heavily on flexible fitting strategies that involve computationally adjusting pre-existing models to match low-resolution density features. While such

methods are sometimes acceptable for generating hypotheses, they cannot serve as conclusive evidence for novel interaction interfaces. The study does not provide key validation metrics such as cross-validation using half-maps, map-to-model correlation coefficients, or local-resolution assessments that would indicate whether the fitted models meaningfully reflect the underlying data.

Even more problematic is the inherent heterogeneity of BAX oligomers, which has been highlighted in multiple independent studies. BAX assemblies are dynamic, size-variable, and structurally heterogeneous, posing significant challenges for cryo-EM reconstruction. The paper does not convincingly address these complexities nor does it demonstrate that the reconstructed densities correspond to a single, well-defined oligomeric species. Without such assurance, the structural model presented remains speculative. The cryo-EM dataset lacks the robustness required to substantiate the claim that the anti-apoptotic dimer interacts with a discrete, structurally consistent interface on BAX oligomers.

3.3. Live-Cell and Fixed-Cell Assays for Mitochondrial Outer Membrane Permeabilization

The cellular component of Newman *et al.*'s experimental framework aims to demonstrate that the anti-apoptotic dimer inhibits BAX-mediated mitochondrial permeabilization in living cells. To support this claim, the authors use confocal microscopy, mitochondrial potential dyes, cytochrome c immunostaining, and caspase activation assays. While these methods are widely used to assess apoptosis dynamics, the study's execution and reporting raise several issues.

One limitation concerns the exclusive reliance on overexpression systems. High-level expression of BAX, anti-apoptotic family members, or engineered dimers can artificially drive interactions by altering stoichiometry or mislocalizing proteins within the mitochondrial membrane, potentially leading to non-physiological interactions and confounding the interpretation of results. The authors do not provide experiments showing endogenous protein levels, nor do they validate whether the overexpressed dimer mirrors endogenous behavior, should it exist physiologically. This restricts generalizability and increases the possibility that the observed interactions are artefacts of supraphysiological expression.

Another concern is the temporal resolution of MOMP measurements. The study frequently quantifies MOMP using endpoint assays, rather than continuous live-cell monitoring capable of capturing the kinetics of pore formation and inhibition. Without time-resolved data, it is difficult to determine whether the dimer truly inhibits pore expansion or simply delays its kinetics. Moreover, the imaging datasets lack quantification of mitochondrial morphology, membrane potential variance, and

cell-to-cell heterogeneity—critical variables when interpreting mitochondrial events. The absence of standardized normalization across replicates further complicates interpretation.

The fixed-cell cytochrome c staining experiments suffer from limited quantification and do not convincingly distinguish between partial and complete MOMP. As a result, the cellular evidence does not definitively support the mechanistic conclusion that oligomeric BAX pores remain regulatable after activation.

3.4. Genetic Manipulations, Mutational Scanning, and Their Interpretive Limitations

Newman *et al*.[5] employ genetic approaches such as siRNA-mediated knockdowns, CRISPR-based deletions, and site-directed mutagenesis to explore how alterations in BAX or the anti-apoptotic dimer affect their interaction. While such experiments can be powerful tools, their execution in the study reveals interpretive weaknesses.

The mutational scanning analyses fail to provide a cohesive structure–function framework. Mutants are introduced in both the dimer and BAX, but their effects on binding, oligomerization, and apoptosis are not systematically aligned with structural predictions. Without quantitative binding affinities, the mutagenesis data cannot be meaningfully integrated into the proposed interface model. Additionally, the lack of rescue experiments—where a loss-of-function mutation is complemented with a compensatory mutation—limits the mechanistic clarity of the mutational results.

The study also lacks rigorous genetic validation using cells deficient in both BAX and BAK. Such cells would serve as an essential baseline for determining whether the dimer acts specifically on BAX or engages other pro-apoptotic factors. Instead, the authors present experiments in single-knockout backgrounds that do not adequately isolate the mechanism.

Collectively, the genetic approaches used in the study generate interesting observations but fall short of supporting the mechanistic claims. They suffer from limited quantification, incomplete structural alignment, and insufficient control conditions.

4. Conceptual Strengths and Points of Innovation

4.1. A New Regulatory Layer for Active BAX Rather than Latent BAX

One of the most striking conceptual contributions of Newman *et al*.[5] is their attempt to reposition anti-apoptotic regulation at a stage beyond what has traditionally been considered the irreversible commitment step in apoptosis. Apoptotic regulation has

historically been framed as a contest at the level of activation, sequestration, or neutralization of latent pro-apoptotic factors such as BAX and BAK. Once activated BAX reaches the mitochondrial outer membrane and transitions into its oligomeric pore-forming state, existing models argue that the regulatory landscape narrows dramatically, leaving little opportunity for upstream controls to intervene. Newman *et al*.[5] challenge this entrenched assumption by proposing that the anti-apoptotic dimer interacts directly with activated, oligomeric BAX to alter pore formation or function.

This conceptual reframing introduces a dynamic regulatory architecture in which BAX activity remains modulatable even after oligomerization. If substantiated, such flexibility would force a reevaluation of the temporal boundaries within which regulation of the intrinsic apoptotic pathway occurs. It would imply that apoptosis is not a binary system abruptly shifting from latency to commitment, but rather a continuum of reversible regulatory decision points. The idea that cells may exert fine control over BAX-mediated membrane permeabilization after pore nucleation carries potentially transformative implications for both cellular physiology and therapeutic strategies.

4.2. Evidence Supporting Post-Oligomerization Modulation of Apoptosis

The study provides several lines of evidence that gesture toward the possibility of such late-stage regulation. Although imperfect, the biochemical binding assays show that the anti-apoptotic dimer associates more strongly with oligomeric BAX than with monomeric or inactive forms. In principle, this selective binding hints at an interface or conformational feature present only after BAX activation. The cryo-EM data, though limited in resolution, depict densities suggestive of a multicomponent protein complex that the authors interpret as representing a stable association between active BAX and the dimer. Even the cellular assays—however confounded by overexpression—suggest a reduction in cytochrome c release and caspase activation in the presence of the dimer, implying some functional inhibition downstream of BAX activation.

It is important to note that these findings do not conclusively demonstrate the claimed mechanism, but they do raise the intriguing possibility that oligomerized BAX remains structurally dynamic or partially reversible. The concept of "pore plasticity" has gained traction in recent years, with evidence suggesting that BAX pores can expand, contract, or exist in multiple intermediate states. Newman *et al*. attempt to position their dimer-mediated inhibition within this framework, theorizing that the dimer binds at a point when pores are sufficiently formed to be functional yet still structurally malleable. Although speculative, this conceptual

alignment with emerging models of pore plasticity gives the study a degree of conceptual resonance.

4.3. The Paper's Position in the Broader Apoptosis Literature

From a theoretical standpoint, the study touches upon long-standing questions in apoptosis research that have remained unresolved. One such question concerns whether MOMP is universally irreversible or modifiable under specific conditions. Historically, the notion of "*minority MOMP*" or partial cytochrome c release has been invoked to explain survival after submaximal apoptotic stimuli, but the mechanistic basis for such phenomena remains unclear. Newman *et al*. attempt to attribute such incomplete commitment events to direct modulation of BAX pores, thereby offering a mechanistic model for a phenomenon previously described in mainly phenomenological terms.

Furthermore, the study indirectly engages with debates surrounding the role of anti-apoptotic proteins as regulators of membrane integrity rather than pure sequestration factors. A growing body of work suggests that anti-apoptotic proteins influence membrane biophysics, including lipid domain organization, cristae remodeling, or mitochondrial morphology. Newman *et al*.[5] extend this model by framing the anti-apoptotic dimer as a protein that interacts with and modifies the stability of active pores themselves. If correct, this would blur the traditional distinction between upstream regulatory proteins and downstream effector complexes, situating anti-apoptotic regulation as an integrated component of membrane remodeling.

The study's proposed mechanism might also complement recent observations that BAX pores can be influenced by mitochondrial proteins previously thought to be tangential to classical apoptosis regulation. The emerging view of the mitochondrial outer membrane as a dynamic environment, whose physical properties influence pore formation, resonates with the idea that regulatory actors may operate at later stages. Thus, Newman *et al*. tap into a growing interest in the spatial and temporal complexity of apoptosis.

4.4. Pharmacological Implications for Cancer, Degeneration, and Tissue Injury

If the mechanism proposed by Newman *et al*.[5] were validated, the therapeutic implications would be profound. One major implication concerns cancer therapy. Many cancer cells evade apoptosis through overexpression of anti-apoptotic proteins such as BCL-2 and MCL-1. If these proteins or their engineered dimers can inhibit even fully activated BAX, then tumors may possess more powerful resistance strategies than previously recognized. This would necessitate rethinking current

BH3 mimetic strategies, which assume that release of BAX from sequestration results in irreversible pore formation. If pore formation remains modulatable post-oligomerization, then BH3 mimetics may require co-targeting with agents that block late-stage anti-apoptotic interference.

Conversely, excessive apoptosis contributes to neurodegeneration, ischemia-reperfusion injury, and inflammatory tissue damage. If the anti-apoptotic dimer indeed stabilizes sublethal BAX assemblies, then pharmacologically mimicking this activity might provide a novel therapeutic avenue for conditions where BAX-mediated mitochondrial dysfunction is a key driver of pathology. Current anti-apoptotic drugs focus on preventing BAX activation rather than modulating its active forms; the new model suggests that targeting pore dynamics could be a viable strategy.

The study's proposal might also intersect with the burgeoning field of controlled cell death modulation for regenerative medicine or precision cell elimination. Synthetic biology approaches increasingly attempt to design switches that modulate apoptosis thresholds. A mechanism that allows intervention after pore formation could significantly expand the design space for therapeutic control circuits.

Despite these theoretical advantages, therapeutic extrapolation is premature given the methodological gaps present in the study. The lack of rigorous *in vivo* validation, physiological expression data, and structural clarity limits the confidence with which one might integrate the findings into drug development contexts. Nevertheless, by proposing that apoptosis remains modulatable at a late stage, Newman *et al*. open a conceptual pathway toward pharmacological strategies that have previously been dismissed as impossible within the classical apoptosis paradigm.

5. Critical Weaknesses in Study Design and Interpretation

5.1. Inconsistencies in the Working Model across Figures

One of the most striking issues in Newman *et al*.[5] is the inconsistency in the proposed mechanism across the figures. The authors maintain that the anti-apoptotic dimer inhibits oligomeric BAX, yet the biochemical, structural, and cellular data do not cohere into a unified mechanistic framework. For instance, the biochemical assays in early figures suggest that the dimer binds preferentially to oligomeric BAX, but the particular oligomeric states represented in each assay are neither standardized nor rigorously quantified. Some experiments appear to involve detergent-assembled oligomers, others use liposome-inserted BAX, and still others rely on overexpressed mitochondrial BAX in cells. These systems differ substantially

in conformation and stability, making it problematic to draw mechanistic conclusions across them.

Furthermore, the structural cartoon presented in the final figure depicts a highly specific interaction interface, yet the cryo-EM maps shown earlier in the paper do not convincingly support such precision. The working model suggests a single, discrete inhibitory mode, but the heterogeneity of the datasets and the limited clarity of interaction surfaces undermine that claim. These inconsistencies create a narrative tension: the authors present a clean conceptual model, but the underlying data appear fragmented, speculative, or insufficiently aligned with that model. As a result, the story becomes more conjectural than empirical.

5.2. Insufficient Controls for BAX Activation State and Stoichiometry

A major interpretive weakness is the lack of rigorous control over the activation state of BAX in many of the experiments. BAX exists in multiple conformational states, from inactive monomers to activated intermediates and fully oligomerized pore-forming structures. The authors claim selective interaction with oligomeric BAX, but they do not systematically distinguish between these states using established tools such as conformation-specific antibodies, limited proteolysis, crosslinking assays, or fluorescence-based activation reporters. Without such controls, it remains unclear whether the dimer truly recognizes only active, pore-forming BAX or whether it binds earlier intermediates that accumulate under the experimental conditions.

Stoichiometry is another key issue that the paper does not adequately address. The molar ratios of the dimer relative to BAX vary widely across assays and are often far from physiological levels. In overexpression systems, the dimer may be present at concentrations orders of magnitude higher than endogenous BAX, raising the likelihood of non-specific interactions. The authors provide no quantitative binding affinity measurements, leaving the strength, specificity, and functional relevance of the interaction ambiguous. In reconstitution experiments, oligomeric BAX levels are not quantified, and the degree of oligomerization is inferred rather than measured. These stoichiometric uncertainties fundamentally compromise the ability to draw mechanistic conclusions.

5.3. Under-Documented Validation of Protein Conformations and Purity

The study relies heavily on recombinant proteins, yet provides insufficient documentation of their purity, folding state, and functional competency. SDS–PAGE gels and chromatograms shown in the Extended Data are inadequate substitutes for

the biophysical validation typically required for structural and mechanistic studies of this kind. The purity of BAX is especially important because contaminants or partially folded species can unpredictably influence oligomerization outcomes. Similarly, the authors do not verify that the anti-apoptotic dimer adopts a physiologically relevant conformation. Without circular dichroism, thermal shift assays, NMR spectra, or activity-based validation, it is impossible to know whether the recombinant dimer reflects the functional form posited in the model.

Additionally, the choice of detergents used in reconstitution poses interpretive challenges. Detergents can destabilize or artificially stabilize oligomeric states of BAX, and small variations in concentration can disproportionately influence assembly outcomes. The authors provide only limited methodological detail, leaving essential parameters—such as lipid-to-protein ratios, membrane composition, or detergent concentrations—unexplained. These omissions raise the possibility that observed binding events reflect artefacts of the preparation rather than intrinsic biochemical properties.

5.4. Logical Gaps between Structural Claims and Cellular Outcomes

Perhaps the most significant interpretive weakness in the paper lies in the logical discontinuity between the structural mechanistic claims and the cellular datasets. The cryo-EM models proposed by the authors imply a specific and well-defined inhibitory interface, yet the structural data lack the resolution needed to substantiate such a claim. The cellular experiments, meanwhile, rely on endpoints rather than detailed kinetic analyses, making it difficult to connect molecular interactions to functional consequences. These gaps in logical continuity compromise the internal coherence of the study.

For example, the authors claim that the dimer selectively inhibits pore expansion rather than initial pore formation. However, the imaging data presented do not possess the temporal or spatial resolution required to differentiate between these two steps. Mitochondrial permeabilization is a highly dynamic event and requires live, high-frame-rate imaging to resolve mechanistic details. The authors instead rely on static cytochrome c staining or low-frequency time-lapse imaging, limiting interpretive depth. These methodological limitations mean that the study's mechanistic claims outstrip its evidentiary foundation.

Additionally, the authors do not consider alternative interpretations of the data, such as indirect effects on mitochondrial membrane composition or metabolic remodeling. Anti-apoptotic proteins are known to influence mitochondrial dynamics, and changes in membrane tension or lipid microdomains could indirectly influence BAX pore formation. The paper does not address these possible

explanations. Instead, the authors assume direct binding and functional inhibition of oligomeric BAX as the primary mechanism without establishing the exclusivity of that interpretation.

5.5. Failure to Provide Orthogonal Validation of Key Claims

Mechanistic studies of this magnitude typically require multiple independent lines of evidence to reinforce central claims. In this regard, Newman *et al*.[5] fall short. They rely heavily on cryo-EM and overexpression-based cellular assays, without complementing these approaches with orthogonal validation methods. For instance, biochemical crosslinking could corroborate the proposed interface between the dimer and BAX. Single-molecule fluorescence experiments could assess pore dynamics and dimer interactions in real time. Genetic approaches could evaluate the impact of dimer depletion or mutation under endogenous expression conditions. None of these critical approaches are deployed.

Furthermore, the study lacks *in vivo* validation. Although *in vitro* and cell-based systems can suggest mechanistic possibilities, mitochondrial apoptosis is deeply contextual and influenced by metabolic, structural, and physiological factors that cannot be recapitulated in simplified models. Without *in vivo* data, it remains uncertain whether the proposed interaction is remotely physiologically relevant or simply an artefact of overexpression and reconstitution.

5.6. Conceptual Overextension of Weak Evidence

Finally, the paper overextends its conclusions beyond what the evidence supports. The authors assert that the anti-apoptotic dimer reveals a new universal regulatory mechanism of apoptosis, yet their evidence derives from limited systems, incomplete structural models, and ambiguous cellular responses. The claim that apoptosis remains regulatable at late stages is far-reaching, and if true, would require extensive validation across cell types, stress contexts, and physiological conditions. The data presented fall well short of such a threshold.

6. Figure-by-Figure Critique of Main Figures

6.1. Figure 1: Biochemical Evidence for Dimer Binding to Oligomeric BAX

Figure 1 serves as the biochemical foundation for the study's central claim, yet it displays multiple methodological and interpretive deficiencies. The pulldown assays show bands suggesting that the anti-apoptotic dimer associates with BAX, but the experimental design lacks clarity on the conformational state of BAX in each condition. The authors claim selective binding to oligomeric BAX, but the gel images do not distinguish monomeric, dimeric, or higher-order species. Without the use of conformation-specific antibodies or well-characterized oligomerization mutants, the

data cannot decisively establish that the interaction occurs only with oligomerized BAX.

Furthermore, the authors do not include negative controls using *BAX* mutants incapable of oligomerization, which would be essential to demonstrate specificity. The detergent-based oligomerization system used in the biochemical assays introduces uncertainty regarding the physiological relevance of the observed interactions. Detergent micelles can artificially promote hydrophobic interactions, creating non-physiological assemblies. The lack of isothermal titration calorimetry, surface plasmon resonance, or quantitative affinity measurements further weakens the argument. Because the pulldown experiments rely entirely on endpoint visualization and lack quantitative normalization, the figure provides suggestive but not mechanistically convincing evidence.

6.2. Figure 2: Structural Interpretation and Cryo-EM Density Ambiguities

Figure 2 presents low- to medium-resolution cryo-EM reconstructions of what the authors interpret as the BAX–dimer complex. However, the density maps in this figure are too ambiguous to support the structural claims made in the text. The models are fitted into diffuse densities that lack clear side-chain discrimination, raising concerns about overinterpretation. The authors manipulate pre-existing models of BAX and the dimer to conform to poorly defined densities through flexible fitting, but such approaches can embed investigator bias and artificially impose structure where none exists.

The purported interface between helices α4 and α6 of BAX and a composite surface on the dimer is not discernible in the density maps shown. The boundaries of the dimer are unclear, with no consistent features across 2D class averages. Substantial heterogeneity is evident in the datasets, yet the authors present a singular mechanistic model without showing 3D variability analysis or alternative reconstructions. The absence of map-to-model cross-validation metrics, Fourier shell correlation curves for local regions, and independent half-map comparisons further undermines the structural interpretation. **Figure 2** thus provides insufficient structural support for the claim that the anti-apoptotic dimer binds activated BAX in a specific manner.

6.3. Figure 3: Mitochondrial Functional Assays and Imaging Limitations

Figure 3 attempts to demonstrate that the dimer functionally inhibits BAX-mediated mitochondrial outer membrane permeabilization in cells. The data rely heavily on mitochondrial dyes, cytochrome c immunostaining, and coarse time-

lapse imaging. The central problem is the lack of temporal resolution necessary to distinguish inhibition of pore expansion from mere delay in pore formation. The authors rely on endpoint staining to infer mechanistic action, but such endpoints are not sufficient to draw conclusions about dynamic pore behavior.

Moreover, the experiments are conducted predominantly in overexpression systems, where BAX levels may be supra-physiological. This raises the possibility that overexpression creates anomalous localization patterns or saturates downstream pathways, confounding interpretation. The quantification presented in the figure lacks normalization across replicates and does not include essential measures such as mitochondrial morphology variance, heterogeneity among cells, or the proportion of cells undergoing partial versus complete MOMP. The cytochrome c staining assays appear to show reduced release in dimer-expressing cells, but without multiplexed staining for mitochondrial integrity or real-time tracking of cytochrome c efflux, the conclusion that pore expansion is inhibited—not simply slowed—is unsubstantiated. **Figure 3** therefore falls short of providing functional evidence that aligns with the structural or biochemical claims.

6.4. Figure 4: Cellular Survival, Caspase Activation, and Rescue Logic

Figure 4 aims to connect the molecular mechanism to downstream apoptotic outcomes, such as caspase activation and cell viability. The figure shows reduced caspase-3 cleavage and increased survival in cells expressing the anti-apoptotic dimer. However, these results are difficult to interpret due to the lack of appropriate controls. Caspase activation is subject to amplification logic that can exaggerate small upstream differences; thus, the observed changes may reflect altered signaling thresholds rather than direct modulation of BAX pores.

Additionally, the authors do not adequately control for off-target effects of dimer overexpression. For instance, anti-apoptotic proteins are known to influence ER stress responses, autophagy, or cellular metabolism, any of which could indirectly diminish caspase activation. The Western blots shown in the figure lack loading controls for mitochondrial content and do not quantify the relative contributions of mitochondrial versus cytosolic caspase activation. The rescue experiments also lack rigor: expressing the dimer in BAX-deficient backgrounds should abolish the observed protection if the mechanism is specific to BAX. The authors provide limited evidence of such specificity. As a result, the causal link between dimer activity and apoptotic suppression remains tenuous.

6.5. Figure 5: Mutant Analysis and Overexpression Artifacts

Figure 5 introduces a series of mutational analyses intended to identify residues critical for the dimer–BAX interaction. However, the mutational logic is not clearly aligned with the structural models proposed earlier. Several mutations are introduced into regions of the dimer or BAX without justification grounded in structural prediction or biochemical evidence. The absence of binding affinity measurements makes it difficult to interpret whether mutations disrupt the interaction or merely destabilize the proteins.

The reliance on overexpression systems is particularly problematic in this figure. Mutant *BAX* constructs displayed in the figure may misfold or mislocalize, yet these possibilities are not systematically evaluated. Similarly, the impact of mutations on mitochondrial targeting or membrane insertion is not assessed. The mechanistic conclusions derived from the mutagenesis data are therefore weak, and the figure serves more as a collection of observations than as a coherent analysis of structure–function relationships.

6.6. Figure 6: Proposed Mechanistic Model and Conceptual Inconsistencies

Figure 6 presents a polished mechanistic model depicting how the anti-apoptotic dimer binds and inhibits oligomeric BAX. However, this model extends far beyond what the data support. The figure portrays a well-defined interface with precise structural features, despite the lack of cryo-EM resolution needed to justify such specificity. It also implies a uniform inhibitory mechanism, yet the heterogeneity seen in oligomeric BAX assemblies contradicts the idea that a single interface could account for the observed effects.

Another issue is the depiction of the dimer as acting exclusively on oligomeric BAX, despite the absence of evidence ruling out interactions with earlier intermediates. The mechanistic steps shown in the model introduce claims not directly tested in the experimental data, such as the stabilization of sublethal pore intermediates or the displacement of BAX monomers during pore expansion. These conceptual leaps undermine the model's credibility. **Figure 6** provides an attractive narrative visualization, but it lacks empirical grounding and may bias readers toward conclusions not justified by the data.

7. Extended Data (ED) Figures: Methodological and Interpretive Problems

7.1. ED Set 1: Protein Purity, Oligomeric State, and Missing Controls

ED Set 1 is intended to demonstrate the quality and molecular state of recombinant BAX and the anti-apoptotic dimer used throughout the study. However, this dataset exposes deeper methodological shortfalls that undermine the reliability of all downstream experiments. The SDS–PAGE gels shown lack the resolution and annotations needed to confirm protein purity. Smearing, unexpected bands, and faint high-molecular-weight species are evident but unaddressed. Such bands could represent degradation products, improperly folded intermediates, or co-purifying contaminants; any of these could affect binding specificity or oligomerization behavior.

More critically, the authors attempt to characterize BAX oligomerization using size-exclusion chromatography, yet they present only partial traces without molecular-weight standards or multi-angle light scattering. Without absolute mass determination, one cannot distinguish between dimeric, tetrameric, or larger oligomeric assemblies. The chromatograms do not clearly demonstrate whether the oligomeric species used in binding assays correspond to membrane-inserted pores or detergent-induced aggregates. Several studies have highlighted how detergents artificially promote or destabilize specific BAX conformations, making it essential to validate oligomeric species under multiple conditions. **ED Set 1** fails to do so.

The dataset also lacks negative controls. No BAX mutants known to abolish oligomerization are included, nor are monomeric or partially activated BAX species characterized. Without these controls, it is impossible to attribute observed interactions to true oligomer binding rather than non-specific associations with misfolded protein.

7.2. ED Set 2: Cryo-EM Class Averages and Unresolved Conformations

ED Set 2 is intended to support **Figure 2** by showing additional cryo-EM 2D class averages, particle stacks, and preliminary reconstructions of the BAX–dimer complex. Instead, the dataset highlights the fundamental ambiguity of the structural claims. The 2D classes presented lack clear secondary-structure features, with most appearing as amorphous or partially smeared densities. The authors nevertheless claim that these classes represent distinct orientations of the complex, but the lack of visible helical elements or consistent morphological features renders that claim highly questionable.

The dataset also shows evidence of severe particle heterogeneity, which the authors mention but do not adequately address. The absence of variability analysis, 3D classification, or alternative reconstructions prevents meaningful interpretation of the underlying structural states. In heterogeneous systems such as oligomeric BAX pores, state segregation is crucial. Without it, any averaged density risks conflating multiple structurally distinct species into an artefactual composite.

Furthermore, local-resolution maps are not provided, obscuring the degree to which certain regions of the density may be unreliable. The authors rely heavily on flexible fitting, but do not demonstrate model-to-map correlation or provide difference maps showing unassigned density regions. These omissions weaken the structural argument and raise concerns that the interpreted interface between the dimer and BAX may reflect model bias rather than empirical evidence.

7.3. ED Set 3: Cell Line Variability and Experimental Reproducibility

ED Set 3 attempts to show that the inhibitory effect of the anti-apoptotic dimer on BAX-mediated apoptosis is consistent across multiple cell lines. The intent is valuable, yet the dataset fails to provide the methodological depth necessary to support reproducibility claims. Several cell lines used in the assays are presented without key metadata, such as passage number, growth conditions, or transfection efficiency. These factors can dramatically alter mitochondrial physiology and apoptotic sensitivity, making their omission problematic.

The mitochondrial permeabilization assays displayed in this dataset suffer from inconsistent staining, uneven illumination, and poorly controlled acquisition settings. This heterogeneity makes quantitative comparison across cell lines unreliable. The authors do not normalize mitochondrial mass, membrane potential, or cytosolic volume, all of which influence readouts of cytochrome c release or dye retention.

Another major issue is the lack of replicates. The authors present representative images but offer minimal statistical treatment. There is no indication of how many cells or fields of view were analyzed, whether blinding was implemented, or how variability was quantified. Without rigorous statistical annotation, **ED Set 3** cannot meaningfully support the claim that the dimer's inhibitory effect generalizes across cell types.

7.4. ED Set 4: Missing Negative Controls and Misinterpreted Baselines

ED Set 4 is meant to provide additional biochemical and cellular validations, yet it features some of the most critical omissions in the entire paper. Several key

negative controls are missing. For example, the authors do not test whether the dimer interacts with BAX mutants lacking helix α9, which would clarify whether membrane insertion is necessary for the proposed interaction. They also fail to examine whether monomeric versions of the anti-apoptotic protein bind BAX, a crucial test for demonstrating the specificity of dimerization-dependent inhibition.

The dataset further contains control experiments with scrambled or inactive variants of the dimer, but these controls are poorly designed. In several cases, the scrambled variants exhibit partial binding or functional effects, contradicting claims of specificity. The authors dismiss these anomalies as background noise rather than addressing what they imply about non-specific interactions or experimental artefacts.

Additionally, the baseline comparisons used to interpret inhibition are flawed. The authors frequently compare dimer-treated conditions to overstimulated apoptosis conditions rather than appropriate matched controls. As a result, differences in cytochrome c release or caspase activation may reflect variations in apoptotic stimulus rather than genuine inhibitory activity. The lack of precise baseline modeling also complicates interpretations of partial MOMP, a phenomenon subject to substantial biological variability.

ED Set 4 also lacks essential statistical detail. The absence of replicates, error bars, and analysis of variance leaves many plots uninterpretable. This dataset was clearly intended to bolster weaker aspects of the main figures, yet it instead magnifies concerns about methodological rigor.

7.5. ED Set 5: Ambiguous Kinetic Data and Low-Resolution Time Courses

Another problematic dataset is **ED Set 5**, which includes time-lapse imaging of mitochondrial permeabilization. The authors use these data to claim that the dimer delays or inhibits pore expansion. However, the imaging frequency is too low to capture the rapid kinetics of MOMP. Events that occur on the scale of seconds to minutes cannot be properly resolved with imaging intervals approaching several minutes. This temporal undersampling introduces significant interpretive ambiguity.

The authors also fail to provide pixel-level quantification of cytochrome c diffusion, mitochondrial fragmentation, or mitochondrial–cytosolic gradient changes. Without rigorous kinetic analysis, the suggestion that the dimer acts specifically on pore expansion rather than pore initiation is unsupported. **ED Set 5** thus cannot substantiate the mechanistic step the authors attribute to the dimer.

8. Supplementary Figures and Tables: Technical Documentation Gaps

8.1. Supplementary Structural Models and Unjustified Fitting Assumptions

The **Supplementary Figures** include several structural models intended to provide additional clarity on how the anti-apoptotic dimer engages oligomeric BAX. However, these models suffer from both overinterpretation and inadequate methodological transparency. Many of the models are generated using computational fitting tools that impose pre-existing structural templates onto low-quality density regions. The authors do not justify their template choices, nor do they evaluate alternative fits. This lack of alternative modelling contrasts with best practices in modern cryo-EM, where ambiguous densities are often explored via multiple fitting strategies or left uninterpreted.

The Supplementary structural models frequently display atomic-level detail that the underlying density maps cannot support. For example, side-chain identities, hydrogen bonds, and salt-bridge interactions are illustrated as if they were derived from high-resolution crystallographic data. In reality, the cryo-EM maps provided have insufficient local resolution to support such precise annotation. The failure to distinguish between speculative and empirically grounded modeling renders these figures misleading. Moreover, no validation metrics are provided, such as MolProbity scores, map-to-model fits, or cross-validation statistics. This absence of validation exacerbates the concern that the models reflect artistic interpretation more than structural reality.

8.2. Supplementary Biochemistry: Stoichiometry and Interaction Ambiguity

Several Supplementary biochemical assays attempt to quantify the stoichiometry of dimer–BAX binding or assess the effect of mutations on interaction strength. Yet these experiments are inadequately reported and lack the rigor required to support quantitative conclusions. For instance, the authors present band-intensity quantification from pulldown assays, but band intensities are not normalized to input controls or loading standards. This makes it impossible to evaluate relative binding affinities or stoichiometric ratios.

The Supplementary pulldown assays also fail to incorporate competition experiments that would test whether the dimer directly competes with established BAX interaction partners. Without such tests, the possibility remains that the observed binding reflects indirect association mediated by detergent micelles or protein aggregates rather than genuine molecular recognition.

In several **Supplementary Figures**, the authors claim that specific mutations reduce binding, but the protein expression levels and folding states of these mutants are not shown. Unfolded or partially folded proteins often lose binding capacity for reasons unrelated to interface disruption. Because the authors do not provide circular dichroism, protease sensitivity assays, or thermal denaturation profiles, it is impossible to determine whether functional reductions reflect structural perturbation or global destabilization. As a result, the Supplementary biochemical data lack interpretive clarity.

8.3. Supplementary Cell-Biology Experiments: Missing Metadata and Incomplete Reporting

The Supplementary cell-biology figures aim to provide further validation of the cellular consequences of dimer expression, but they suffer from insufficient metadata, incomplete reporting, and poor experimental standardization. Crucial methodological details are missing, such as transfection efficiencies, expression levels, mitochondrial mass normalization, and time intervals for imaging. These omissions make it impossible to assess reproducibility or compare results across experimental conditions.

Several images presented in the Supplementary material display uneven staining or inconsistent imaging settings. The authors do not document how imaging thresholds were chosen or whether analyses were performed in a blinded manner. Without such methodological safeguards, image-based quantification is vulnerable to confirmation bias. Moreover, many of the Supplementary microscopy figures lack scale bars, making it difficult to evaluate mitochondrial morphology or cytochrome c distribution patterns.

The Supplementary text also lacks important contextual information. For instance, some cell lines used in the Supplementary figures differ from those in the main figures, yet the authors provide no explanation for these choices or justification for mixing heterologous systems. These inconsistencies raise concerns about the reproducibility of the findings and the interpretive linkage between cellular observations and the proposed molecular mechanism.

8.4. Supplementary Statistical Files: Inadequate Annotation and Lack of Raw Data

The statistical Supplementary tables are among the weakest components of the study. These tables include summary statistics for several key assays but do not provide essential raw data, such as individual replicates, error measurements, or exact *p*-values. Many entries merely state significance thresholds (for example, "$P < 0.05$") without specifying the statistical test used or the distributional assumptions

underlying those tests. This lack of detail is inconsistent with modern standards for methodological transparency in high-impact journals.

In some cases, the Supplementary tables appear to aggregate data from different experiments without clarifying whether technical replicates or biological replicates were used. This distinction is critical because pooling technical replicates can artificially inflate perceived reproducibility. Furthermore, the authors do not disclose whether multiple-hypothesis correction was applied to analyses involving large datasets, such as proteomics or transcriptomics screens purportedly used to confirm the absence of global transcriptional changes upon dimer expression. Without appropriate corrections, false positives or inflated significance values become likely.

A more serious issue is the absence of raw datasets for key results. The Supplementary material does not include uncropped gels, original microscopy image series, or complete cryo-EM micrographs. Without such data, independent validation becomes impossible. Many journals now require raw data as standard practice for structural and cell-biology studies, and the absence of such materials raises questions about whether the results presented are selectively curated.

8.5. Supplementary Methods: Ambiguous Reconstitution Procedures and Incomplete Controls

The **Supplementary Methods** section is intended to provide detailed descriptions of the reconstitution, imaging, and biochemical workflows but instead reveals significant methodological ambiguity. The authors provide high-level summaries rather than precise experimental conditions. For example, lipid compositions for liposome-reconstitution assays are described vaguely, without specifying molar ratios, lipid sources, or purification methods. Small variations in membrane composition can drastically alter BAX insertion and pore formation, making such omissions consequential.

Additionally, detergent concentrations and buffer compositions for oligomerization assays are imprecisely reported, lacking important details such as pH, ionic strength, or temperature. These parameters influence protein conformation and stability and are essential for reproducibility. The Supplementary Methods also omit the rationale for specific choices, such as why certain detergents were used or why certain cell lines were chosen for specific experiments.

The absence of detailed control conditions further weakens this section. For example, the authors do not report whether BAX oligomerization was tested under conditions mimicking physiological mitochondrial membranes, nor do they assess whether the dimer can bind BAX on native mitochondria isolated from cells. These

gaps leave open the possibility that the observed interactions reflect non-physiological experimental designs.

9. Biological and Mechanistic Implications

9.1. Reconciling Anti-Apoptotic Dimer Activity with Established BAX Regulation Models

The proposition that an anti-apoptotic dimer can inhibit oligomeric BAX introduces a major conceptual disruption to the established hierarchy of mitochondrial apoptosis. For decades, models of BAX regulation have followed a sequential architecture: BH3-only proteins initiate activation, anti-apoptotic proteins modulate activation thresholds by sequestering activators or inactive BAX, and BAX oligomerization marks a decisive commitment step. Once pores form, downstream signaling proceeds rapidly, culminating in cytochrome c release and caspase activation. Under this framework, post-oligomerization regulation is largely absent. Newman *et al*. challenge this paradigm by arguing that BAX pores remain modifiable after formation and that this new anti-apoptotic activity can reverse or interfere with the consequences of activation.

To reconcile this claim with classical models, several assumptions would need to be revisited. First, BAX oligomers would need to possess structural plasticity far greater than previously recognized. Although recent work has suggested that pore expansion and contraction occur, these transitions are hypothesized to involve subtle conformational rearrangements rather than major changes in protein–protein interaction networks. Newman *et al*.'s model[5] implies the latter: that pores contain accessible surfaces capable of being bound and actively remodeled by regulatory proteins. Second, anti-apoptotic proteins are not generally believed to localize in close proximity to mature BAX pores. Their known distribution and membrane affinities do not easily explain the positioning required for engagement with oligomeric BAX assemblies. Without direct evidence of such colocalization under physiological conditions, the model challenges established spatial constraints in mitochondrial apoptosis. Because these reconciliations require substantial revisions to long-standing frameworks, they demand much stronger empirical evidence than the study provides.

9.2. Possible Alternative Mechanisms Not Considered by the Authors

The authors focus on a direct inhibition model, but multiple alternative mechanisms could account for the observed effects without requiring direct binding to oligomeric BAX. One plausible mechanism involves indirect modulation of mitochondrial membrane properties. Anti-apoptotic proteins, including engineered

dimers, are known to alter lipid composition, curvature, and membrane microdomain organization. These changes can influence BAX insertion and pore dynamics independently of direct protein–protein interactions. Several studies have shown that mitochondrial membrane tension and cardiolipin distribution modulate pore expansion rates, raising the possibility that the dimer's inhibitory effect arises from membrane remodeling.

Another alternative mechanism involves sequestration of activated but non-oligomeric BAX intermediates. BAX activation is not binary; activated intermediates may exist in multiple conformational states, some of which have been shown to reversibly expose their BH3 regions. If the dimer binds such intermediates rather than bona fide oligomers, the observed results could reflect pre-emptive inhibition rather than post-oligomerization regulation. This interpretation would be far more consistent with established anti-apoptotic protein behavior. The authors do not adequately test this possibility, nor do they use tools such as conformation-specific antibodies, limited proteolysis, or fluorescence reporters to distinguish intermediate states.

A broader alternative involves cellular stress responses triggered by dimer overexpression. Anti-apoptotic proteins have pleiotropic roles beyond apoptosis regulation, including modulation of ER stress, mitochondrial fusion–fission dynamics, and metabolic homeostasis. Any of these pathways could indirectly diminish apoptotic signaling independent of direct BAX inhibition. Because the study lacks transcriptomic, proteomic, or metabolic profiling, such alternative mechanisms cannot be ruled out. These possibilities undermine the exclusivity of the authors' interpretation and weaken the mechanistic certainty they assert.

9.3. How the Proposed Mechanism Fits within MOMP Threshold Theory

Mitochondrial outer membrane permeabilization is traditionally considered the decisive point of no return in apoptosis, yet several models—such as minority MOMP, threshold modulation, and sublethal mitochondrial permeabilization—have suggested that cells can experience partial MOMP and survive. However, these phenomena have been attributed to insufficient oligomerization, heterogeneous mitochondrial susceptibility, or downstream modulation of caspase activity, rather than reversal of pore formation.

Newman *et al*.[5] attempt to integrate their mechanism into MOMP threshold theory by suggesting that the anti-apoptotic dimer modulates the proportion of mitochondria undergoing full permeabilization. If true, this would imply that BAX pores can be halted, stabilized in sublethal states, or partially dismantled. Yet no direct evidence is presented for mitochondrial heterogeneity in pore states, nor do

the authors provide real-time kinetic analyses needed to demonstrate stabilization of partial pores. Without quantitative modeling of pore size, pore number, or mitochondrial susceptibility distributions, the study does not convincingly connect the proposed mechanism to threshold theory.

Moreover, threshold models typically involve upstream factors—activation levels of BH3-only proteins, anti-apoptotic sequestration capacity, or mitochondrial structural variability—not downstream remodeling of fully activated pores. This makes Newman *et al.*'s proposal a conceptual outlier. If pores remain regulatable after formation, threshold theory would need to incorporate an entirely new decision point, effectively subdividing MOMP itself into multiple mechanistic phases. Such a revision would represent a major theoretical change, yet the study does not provide the comprehensiveness required to justify such a shift.

9.4. Implications for Drug Development and Synthetic Apoptosis Modulators

If the inhibitory mechanism were accurate, it would carry notable implications for pharmacology. First, cancer therapies based on BH3 mimetics—designed to neutralize anti-apoptotic proteins and promote BAX activation—would need reevaluation. If anti-apoptotic dimers can suppress BAX after oligomerization, then BH3 mimetics may be insufficient to guarantee apoptosis in tumors overexpressing such dimers. This could partially explain resistance to BH3 mimetics in certain cancers, though this connection remains speculative without direct evidence of endogenous dimer-like activity.

Second, a validated ability to modulate BAX pores post-formation would provide a new therapeutic target class. In neurodegeneration or ischemic injury, where excessive apoptosis contributes to pathology, stabilizing partial pores or inhibiting pore expansion could protect vulnerable cells. This concept aligns with strategies aimed at modulating mitochondrial permeability transition or inhibiting downstream caspase activity. However, without clear demonstration that pore modulation occurs under physiological conditions, therapeutic extrapolation remains premature.

Third, in synthetic biology, mechanisms that allow reversible modulation of apoptosis could be incorporated into engineered cell circuits designed for controlled cell death. Such circuits would benefit from reversible checkpoints downstream of BAX activation, offering finer control than existing systems. Yet again, without firm mechanistic evidence, integrating such concepts into synthetic designs is premature.

Ultimately, the mechanistic uncertainty and methodological gaps weaken the translational potential of the study. Until the central interaction is convincingly demonstrated, any drug-development implications remain hypothetical.

10. Theoretical and Translational Consequences

10.1. Potential Use in Designing BAX-Targeted Therapeutics

If the inhibitory mechanism described by Newman *et al*. were accurate, it would fundamentally redirect therapeutic strategies targeting the mitochondrial apoptosis machinery. Current pharmacological interventions primarily focus on manipulating upstream regulators such as BCL-2, BCL-XL, or MCL-1, largely ignoring BAX itself as a viable drug target due to its structural dynamism and deep integration into the mitochondrial outer membrane. The authors' proposed mechanism suggests that BAX remains regulatable even in its active, oligomeric state, implying that drugs targeting pore-formed BAX might be feasible. Such an approach would challenge long-standing beliefs that BAX pores, once formed, are irreversible and difficult to access.

Therapeutic agents designed to stabilize partial pores, reverse pore expansion, or promote disassembly could be conceptualized based on the framework proposed by the study. In principle, this could provide new strategies to halt apoptosis in degenerative conditions or enhance apoptosis in malignancies where pore formation is incomplete or inefficient. However, such innovations require a far more rigorous mechanistic foundation than the current study provides. Without conclusive evidence for the existence, structure, and physiological relevance of post-activation modulation, basing drug design on this model risks generating therapeutics that target artefactual or non-physiological interactions.

Moreover, targeting oligomeric BAX would necessitate small molecules or biologics capable of penetrating mitochondrial membranes, navigating the complex lipid environment, and engaging flexible, heterogeneous protein assemblies. These challenges highlight the need for substantial foundational work before therapeutic translation becomes credible.

10.2. Risks of Misinterpreting Structural Interaction Data

The structural inferences presented by Newman *et al*. carry significant risks of misinterpretation. Cryo-EM maps lacking sufficient resolution are vulnerable to overfitting, model bias, and subjective interpretation. If broad adoption of the study's conclusions occurs without appropriate caution, researchers could inadvertently misuse low-resolution structures to justify speculative mechanistic models. This risk extends beyond apoptosis research and into structural biology itself, where interpretive rigor is essential.

Incorrect perception of a stable, well-defined interface between the anti-apoptotic dimer and oligomeric BAX could skew subsequent investigations, encouraging the development of therapeutics or academic hypotheses built on structurally unsound premises. Historically, the field has witnessed similar issues: early low-resolution structures of apoptosome complexes, inflammasomes, and mitochondrial carriers led to speculative mechanistic narratives later contradicted by higher-resolution studies. The same risk applies here unless independent reconstructions, orthogonal validation techniques, and higher-resolution data corroborate the interaction. Misinterpretation could also mislead computational modeling studies, which often rely on structural templates that must be accurate to guide drug discovery or molecular dynamics simulations.

In essence, the premature acceptance of speculative structural data risks creating a conceptual edifice that cannot be sustained once more rigorous analyses are performed. This would not only affect the credibility of the present study but could also distort the trajectory of apoptosis research.

10.3. Broader Impact on Cell-Death Research Paradigms

Accepting the mechanism proposed by Newman *et al*. without decisive evidence would have profound implications for how the scientific community conceptualizes apoptosis. It would require rethinking foundational concepts of mitochondrial permeability control, pore dynamics, and commitment to cell death. For example, the widely accepted notion that MOMP represents an irreversible point of no return would be challenged, forcing a re-examination of decades of experimental and theoretical work supporting this concept.

This shift would extend into related fields such as developmental biology, immunology, neurobiology, and tumor biology. Many models of cell fate rely on the binary nature of apoptosis as a decisive eliminative mechanism. If apoptosis becomes reversible at late stages or subject to modulation at the level of activated BAX pores, then interpretations of tissue remodeling, immune cell homeostasis, and cancer progression would require revision. Such re-interpretation necessitates robust mechanistic grounding; otherwise, foundational concepts risk destabilization without a scientifically sound replacement framework.

Furthermore, the study's implications disturb the established dichotomy between upstream regulators (BH3-only and anti-apoptotic proteins) and downstream effectors (BAX and BAK). Merging these layers into a single regulatory continuum would require substantial evidence not yet provided. Without such evidence, the risk is conceptual inflation: introducing theoretical constructs that complicate rather than clarify apoptosis mechanisms.

10.4. Future Directions for Validation, Reproducibility, and Mechanistic Rigor

Given the high conceptual stakes, future work must adhere to rigorous methodological standards to validate or refute the claims made in this study. Addressing the ambiguities in structural data should be a priority. Cryo-EM reconstructions with significantly higher resolution, ideally supported by crosslinking mass spectrometry and site-directed spin labeling, could provide definitive evidence of binding interfaces. Single-molecule fluorescence imaging would enable direct visualization of pore dynamics and test whether pores remain responsive to inhibitory proteins after formation.

Biochemical reconstitution studies must also improve. Using native mitochondrial membranes, well-characterized lipid compositions, and physiologically relevant protein concentrations would clarify whether the dimer–BAX interaction occurs under close-to-native conditions. Implementing detailed stoichiometric analyses, kinetic assays, and thermodynamic measurements would further illuminate whether binding is direct, specific, and functionally significant.

At the cellular level, endogenous expression systems should replace overexpression-driven assays. CRISPR-based knock-in strategies could enable expression of tagged endogenous dimers or BAX variants, reducing overexpression artefacts. Live-cell imaging with high temporal resolution would be crucial for resolving pore initiation, expansion, and inhibition.

Finally, future studies must incorporate broader systems-level analyses. Transcriptomic, proteomic, and metabolomic profiling could reveal whether the dimer induces compensatory stress responses that confound interpretations. *In vivo* models will be essential to determine whether the mechanism operates in physiological settings or represents a cell-culture artefact.

Without these critical validations, translational enthusiasm must remain tempered. Mechanistic rigor, reproducibility, and structural clarity are prerequisites for any paradigm shift in apoptosis biology.

11. Conclusion

11.1. Overall Assessment of Contributions and Limitations

Newman *et al.*[5] present a provocative and ambitious proposal: that an anti-apoptotic dimer can directly inhibit oligomeric BAX, thereby modulating mitochondrial outer membrane permeabilization at a stage traditionally considered irreversible. The conceptual novelty of inhibiting fully activated BAX pores stands out as the study's most compelling contribution. If true, it would fundamentally revise the long-held

notion that BAX oligomerization marks a definitive commitment point in the apoptotic program. The assertion that pore dynamics remain regulatable introduces a potentially transformative perspective on apoptosis, expanding the known landscape of mitochondrial decision-making.

However, despite this conceptual promise, the experimental foundation supporting the claim is insufficiently rigorous. Across biochemical, structural, and cellular findings, the evidence consistently falls short of the standards required to substantiate a paradigm-shifting mechanism. The biochemical pulldown assays do not adequately distinguish between BAX conformational states; the cryo-EM reconstructions lack the resolution and interpretive clarity needed to assign meaningful interfaces; the cellular experiments rely on overexpression systems vulnerable to artefactual interactions; and the mutational analyses lack a coherent structure–function rationale. ED and Supplementary materials amplify these concerns by exposing missing metadata, irregular controls, non-standard statistical practices, and incomplete methodological documentation.

As a result, while the hypothesis introduced by the authors is scientifically stimulating, the current dataset does not support the strength of the mechanistic conclusions drawn. The paper offers intriguing observations rather than definitive insights, and the lack of orthogonal validation impedes its ability to redefine the field's understanding of apoptosis.

11.2. Required Experiments and Standards for the Field Moving Forward

To determine whether the mechanism proposed by Newman *et al*.[5] represents a true regulatory phenomenon or an artefact of experimental design, several methodological upgrades and conceptual clarifications are required. First and foremost, stronger evidence is needed to support the existence of a direct interaction between the anti-apoptotic dimer and activated BAX oligomers. This will require high-resolution structural studies capable of resolving interfaces at atomic or near-atomic precision, ideally corroborated by crosslinking mass spectrometry, site-specific mutagenesis, and orthogonal biophysical measurements. Without such evidence, the structural component of the model remains largely speculative.

Second, future biochemical studies must address stoichiometry, conformational state, and physiological relevance. Native mitochondrial membranes should replace detergent-based systems wherever possible. The use of BAX mutants that define specific activation intermediates will be essential to test whether the interaction is truly oligomer-specific or reflects binding to an earlier activation stage. Quantitative binding assays should replace qualitative pulldown experiments, providing clear dissociation constants, cooperativity measures, and thermodynamic parameters.

Third, cellular studies must avoid overexpression systems and instead rely on endogenous protein levels, knock-in reporters, or inducible expression systems with controlled stoichiometry. Mitochondrial permeabilization must be monitored with high temporal resolution to distinguish between inhibition of pore formation and modulation of pore expansion. Single-cell and single-mitochondrion analyses will be required to evaluate heterogeneity, partial MOMP, and threshold modulation in a biologically meaningful manner.

Fourth, mechanistic alternatives must be evaluated systematically. Potential contributions from membrane remodeling, metabolic reprogramming, stress-response activation, or indirect effects on mitochondrial bioenergetics need to be explicitly tested and ruled out before concluding that a direct inhibitory mechanism operates on oligomeric BAX.

Finally, transparency, reproducibility, and reporting standards must be elevated. All raw cryo-EM micrographs, uncropped gels, original imaging datasets, and full replicate spreadsheets should be publicly available. Supplementary methods must include precise descriptions of buffer compositions, lipid mixtures, transfection conditions, imaging parameters, statistical tests, and computational procedures. Without such transparency, neither peer reviewers nor the broader scientific community can adequately assess the reproducibility or validity of the claims.

Taken together, these steps define a path for evaluating whether oligomeric BAX inhibition represents a genuine biological mechanism or reflects interpretive overreach. Only through stringent mechanistic validation and methodological rigor can the field determine whether the provocative model introduced by Newman *et al*. warrants incorporation into existing frameworks of mitochondrial apoptosis.

Conflicts of Interest Statement

The authors declare no competing financial or personal interests that could influence the analysis, interpretation, or conclusions presented in this commentary. This work represents an independent and objective evaluation of the study by Newman *et al*. and was conducted without any involvement from the authors of the original research or affiliated institutions. The authors have no financial relationships with entities that could be perceived to affect the impartiality of this critique.

Reference

1 Majeed, N. S. *et al.* Multifaceted roles of Bcl-2 family proteins: Regulatory roles in apoptosis, physiological functions, and therapeutic potential. *Curr Med Sci* (2025). https://doi.org/10.1007/s11596-025-00124-1

2 King, L. E., Hohorst, L. & Garcia-Saez, A. J. Expanding roles of BCL-2 proteins in apoptosis execution and beyond. *J Cell Sci* **136** (2023). https://doi.org/10.1242/jcs.260790

3 Gong, Q. *et al.* B-cell lymphoma-2 family proteins in the crosshairs: Small molecule inhibitors and activators for cancer therapy. *Med Res Rev* **44**, 707-737 (2024). https://doi.org/10.1002/med.21999

4 Oltvai, Z. N., Milliman, C. L. & Korsmeyer, S. J. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74**, 609-619 (1993). https://doi.org/10.1016/0092-8674(93)90509-o

5 Newman, C. E. *et al.* Inhibition of oligomeric BAX by an anti-apoptotic dimer. *Cell* (2025). https://doi.org/10.1016/j.cell.2025.10.037