Dynamics of cell-to-cell variability in TRAIL-induced apoptosis explains fractional killing and predicts reversible resistance

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Francois Bertaux, Szymon Stoma, Dirk Drasdo, Gregory Batt*

INRIA Paris-Rocquencourt, 78153 Le Chesnay, France

*Correspondence: gregory.batt@inria.fr

Abstract

Isogenic cells sensing identical external signals can take markedly different decisions. Such decisions often correlate with pre-existing cell-to-cell differences in protein levels. When not neglected, these differences are accounted for in a static manner by assuming randomly-distributed initial protein levels. Capturing the actual temporal fluctuations of protein levels resulting from protein stochastic turnover would be more appropriate. Adopting this dynamical view on noise amounts to recast extrinsic variability into intrinsic noise. Here, we propose a generic approach to merge in a systematic and principled manner signal transduction models with stochastic protein turnover models. When applied to an established kinetic model of TRAIL-induced apoptosis, our approach markedly increased model prediction capabilities. One obtains a mechanistic explanation of yet-unexplained observations on fractional killing and non-trivial robust predictions of the temporal evolution of cell resistance to TRAIL. Our results challenge the role of surviving pathways in cell survival since no TRAIL-induced regulations are needed and strongly suggest that short-lived anti-apoptotic proteins Flip/Mcl1 exhibit large and rare fluctuations. More generally, our results highlight the importance of accounting for stochastic protein turnover to quantitatively understand signal transduction over extended durations, and imply that fluctuations of short-lived proteins deserve particular attention.

Introduction

TNF-Related Apoptosis Inducing Ligand (TRAIL) is a promising therapeutic agent against cancer because it induces apoptosis specifically in tumor cells (Wiley *et al*, 1995; Walczak *et al*, 1999; Johnstone *et al*, 2008). This motivated dozens of clinical trials based on TRAIL-related therapies. However, efficiency was usually limited (Dimberg *et al*, 2013).

Most of the molecular events leading from TRAIL exposure to cell death are known (Johnstone *et al*, 2008). After TRAIL binding to death receptors, initiator caspases are activated, which in turn promote effector caspases activation either directly or via a mitochondrial pathway (Figure S1). In most cells, Mitochondrial Outer Membrane Permeabilization (MOMP) is required to efficiently activate effector caspases. To assess our quantitative understanding several kinetic models have been proposed to describe those biochemical reactions (Albeck *et al*, 2008; Fussenegger *et al*, 2000; Legewie *et al*, 2006; Chen *et al*, 2007; Rehm *et al*, 2009; Neumann *et al*, 2010; Schliemann *et al*, 2011).

Not all cells of an isogenic population die after TRAIL treatment, even at saturating ligand doses. This fractional killing property is widely shared among cell lines and is critical for therapeutical applications (Spencer *et al*, 2009). In addition, surviving cells were shown to be transiently resistant to a second TRAIL treatment. This reversible resistance property was observed in various cell lines and could also have important implications for therapy (Flusberg *et al*, 2013; Flusberg & Sorger, 2013).

Fractional killing is generally thought to result from cross talks between the apoptosis pathway and survival pathways (Falschlehner *et al*, 2007). Indeed, several studies reported that TRAIL induces an up-regulation of some anti-apoptotic proteins, via the activation of survival pathways (such as NF-κB and Akt; Chaudhary *et al*, 1997; Son *et al*, 2010; Sun *et al*, 2011). While fractional killing illustrates cell-to-cell variability in the decision between life and death, variability is also observed among cells that die: they commit to death after a highly variable delay from one another (Rehm *et al*, 2009; Spencer *et al*, 2009; Bhola & Simon, 2009). This variability cannot be explained by differences in TRAIL-induced gene regulation: it is also observed when cells are co-treated with cycloheximide (CHX), an efficient inhibitor of protein synthesis (Spencer *et al*, 2009).

Rather, it was proposed to originate from pre-existing differences in the levels of proteins composing the apoptosis pathway. Indeed, recently divided sister cells died almost synchronously, as expected if protein content is equally shared between daughters and if noise in signaling reactions play a marginal role (Spencer *et al*, 2009). Because of noisy protein synthesis, memory in protein levels between sister cells should only be transient (Sigal *et al*, 2006). Consistently, death synchrony was weaker in older sisters. This explanation was supported by modeling: using a kinetic model (EARM, Albeck *et al*, 2008), Spencer et al. (2009) showed that initial differences in protein levels are sufficient to explain observed variability in death times. Thus, it is likely that protein level fluctuations caused by constitutive protein synthesis noise are responsible for cell-to-cell variability in death time after treatment with TRAIL and CHX.

When cells are treated with TRAIL alone, protein synthesis is not blocked and those fluctuations are potentially able to impact the decision between life and death. To which extent can fractional killing and reversible resistance be explained solely by constitutive noisy protein synthesis, protein-protein reactions and protein degradation? Is it really necessary to account for TRAIL induced activation of survival pathways?

In this work, we addressed this question by developing a refined model of TRAIL-induced apoptosis, in which we model protein synthesis by using simple and well-accepted stochastic models of transcription (Singh *et al*, 2012; Suter *et al*, 2011; Raj *et al*, 2006; Dar *et al*, 2012). To our knowledge, this is the first attempt to systematically account for gene expression noise in a signal transduction model. It enriches the model with a fundamental property: the dynamics of cell-to-cell variability can be investigated. To limit the number of free parameters, motivated by a mathematical analysis, we equipped stable proteins with standard models of protein fluctuations; only the reaction rate values of the fluctuations models assigned to the few short-lived proteins were systematically explored. We found that cell fate

variability in TRAIL-induced apoptosis, including fractional killing, could be quantitatively reproduced when assuming large, rare fluctuations of the short-lived anti-apoptotic proteins Mcl1 and Flip. Importantly, the fitted model was then able to quantitatively predict transient memory prior to TRAIL treatment (transient cell fate inheritance between sister cells) but also in cells that survived TRAIL treatment (reversible resistance). In other words, key dynamical features of cell resistance to TRAIL robustly emerged from model simulations.

Our results show that accounting for protein synthesis noise in the apoptosis pathway is sufficient to explain fractional killing and reversible resistance and therefore challenge the role of survival pathways in TRAIL-induced apoptosis. More generally, they highlight the importance of stochastic protein fluctuations to investigate signal transduction over large time scales and complex signaling contexts, and imply that for any biochemical pathway of interest, short-lived proteins deserve particular attention as they are more prone to exhibit rapid and large variations.

Results

Extended vision of signal transduction pathways

Signal transduction is essentially mediated by interactions between proteins. Native proteins are activated or inhibited by post-translational modifications such as phosphorylation, cleavage or conformational changes. Because of dilution caused by growth and protein degradation, native proteins needs to be continually synthesized to maintain cell ability to transduce signal, resulting in protein turnover.

Signal transduction often takes places within shorter time-scales than protein turnover. Accordingly, signaling transduction processes are generally explained using the set of protein-protein reactions that achieve signal transduction *per se*. However, protein turnover should not be neglected if one studies the long-term behavior (more than one cell generation) of a

cell population. Moreover, when some proteins are subjected to rapid turnover, even the short-term behavior could be affected (Loriaux & Hoffmann, 2013).

Protein synthesis and degradation are also subjected to noise, resulting in fluctuations of protein concentrations in individual cells and in cell-to-cell variability at the population level (McAdams & Arkin, 1997; Raser & O'Shea, 2005). Such variability could have consequences on signal transduction: aside of conventional epigenetic differences (Rando & Verstrepen, 2007), unequal access to ligand molecules or simply noise in signaling reactions, it often contributes importantly to heterogeneous behavior within an isogenic population (Geva-Zatorsky *et al*, 2006; Spencer *et al*, 2009). One approach to account for those differences is to incorporate protein level variability as random initial conditions of an ODE model describing the signaling reactions (Spencer *et al*, 2009; Gaudet *et al*, 2012). However, variability is imposed at time zero and then behavior is deterministic: it is therefore not appropriate to study transduction on long time scales, during which protein levels dynamically fluctuate (Sigal *et al*, 2006).

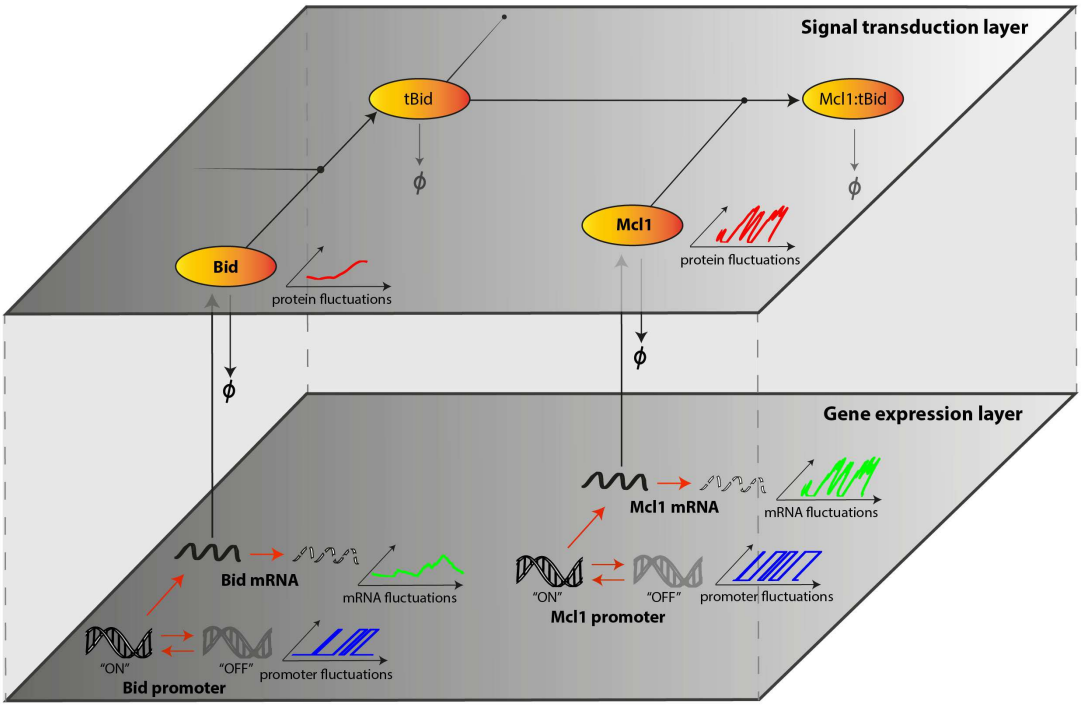

Figure 1: Accounting for stochastic protein turnover in signal transduction pathways Scheme of the modeling approach. Protein-protein interactions mediating signal transduction (signal transduction layer) are modeled by

ordinary differential equations. In parallel, promoter activity changes, mRNA production and degradation (gene expression layer) are seen as stochastic events and generate fluctuations in mRNA levels. This impacts the synthesis rates of the corresponding proteins. Together with protein degradation, it generates fluctuations in protein levels (here shown in absence of transduction). The figure depicts a fragment of the extrinsic apoptosis pathway. Deterministic/stochastic interpretation of chemical reactions is represented with black/red arrows respectively. Empty sets, dashed mRNA, plain/shaded DNA represent protein degradation, mRNA degradation and active/inactive promoter state respectively.

A more natural manner to account for protein level variability is to represent their stochastic synthesis and degradation. Surprisingly, although several studies did account for cell-to-cell differences in protein levels in an extrinsic, static manner via random initial conditions (Spencer *et al*, 2009; Aldridge *et al*, 2011; Stoma *et al*, 2013), and many models of signal transduction considered the effect of noise in protein-protein reactions (Shibata & Fujimoto, 2005; Lapidus *et al*, 2008), no kinetic model of signal transduction pathways considering systematically noise in protein synthesis and degradation has been developed so far.

Here, we propose a modeling approach to account for gene expression noise within kinetic models of signal transduction pathways. Following Singh *et al* (2012), we model protein turnover with stochastic processes describing mRNA level fluctuations, and deterministic processes for protein translation and degradation (random telegraph model, see also Raj *et al*, 2006; Suter *et al*, 2011; Dar *et al*, 2012). These processes are integrated into a kinetic model of protein-protein reactions (Figure 1).

While the rates of such stochastic protein turnover models are rarely directly measurable, their value can be constrained by combining experimental measurements and analytical results (Figure S2). Recently, significant progress has been made on both experimental and theoretical sides to enable this inference approach (Cox *et al*, 2008; Dar *et al*, 2012; Munsky *et al*, 2009; Suter *et al*, 2011; Paszek, 2007; Viñuelas *et al*, 2013). Importantly, we found that for typical protein and mRNA half-lives, a large set of promoter rate combinations lead to similar fluctuations at the protein level, as characterized by protein level variability (coefficient of variation) and mixing time (half-autocorrelation time). Thus, standard

stochastic protein turnover models can provide a good approximation of protein fluctuations for most proteins. However, short-lived proteins necessitate particular attention (Figure S3).

Modeling stochastic protein turnover in TRAIL-induced apoptosis

We applied this approach to TRAIL-induced apoptosis, using EARM kinetic model (Albeck *et al*, 2008; Spencer *et al*, 2009) to describe protein-protein reactions taking place between TRAIL exposure and cell death commitment.

We equipped all native proteins with a default model of stochastic protein turnover, with the exception of Flip and Mcl1, which are known to exhibit very fast turnover (Nijhawan *et al*, 2003; Poukkula *et al*, 2005). Details of model construction are given in Supplemental Information (see dedicated section, Figure S4 and Tables S1-3). Importantly, with the exception of four parameters ("ON" and "OFF" promoter switching rates, mRNA half-life and protein half-life for Flip and Mcl1), all parameters have been constrained based on experimental data. This drastically limited the number of introduced degrees of freedom and made it possible to systematically explore realistic ranges for their values.

To study the influence of stochastic protein turnover on fractional killing and reversible resistance, we sought to confront our model with existing quantitative data about TRAIL-induced apoptosis in HeLa cells. Those experiments, described in detail later, can be classified into two groups based on the type of information they contain: 1) quantification of the variability in cell fate, 2) characterization of the transient memory in cell state. While previous approaches using ODE models with distributions for initial protein levels capturing cell-to-cell variability (Spencer *et al*, 2009; Gaudet *et al*, 2012) are potentially able to reproduce the first type of data, our model including stochastic protein turnover is uniquely positioned to account for both types of data. We adopted the following strategy: first, search for models able to reproduce observations on cell fate variability; and second test whether valid models can robustly predict observed behaviors where transient memory matters.

Stochastic protein turnover models predicts transient memory in cell sensitivity to TRAIL and CHX

Using live-cell microscopy, Spencer *et al* (2009) investigated the fate of hundreds of cells after exposure to TRAIL and CHX (10 ng/mL and 2.5 μg/mL, Figure 2A). All cells undergo MOMP with a highly variable delay (from 2 to 8 hours, Figure 2B). To study cell fate inheritance, the authors also recorded 20 hours before treatment to identify sister cells (Figure 3A). They were found to have highly correlated MOMP times (correlation coefficient close to 1 for recently divided cells, about 0.5 for older sisters - Figure 3B, black curve). Here, the MOMP time distribution provides a quantification of the cell fate variability, while MOMP time correlations between sister cells also give information on the transient memory in cell state. Within our framework, *in-silico* reproduction of those experiments is straightforward (Figures 2D and 3C), enabling us to investigate possible origins of transient cell fate inheritance.

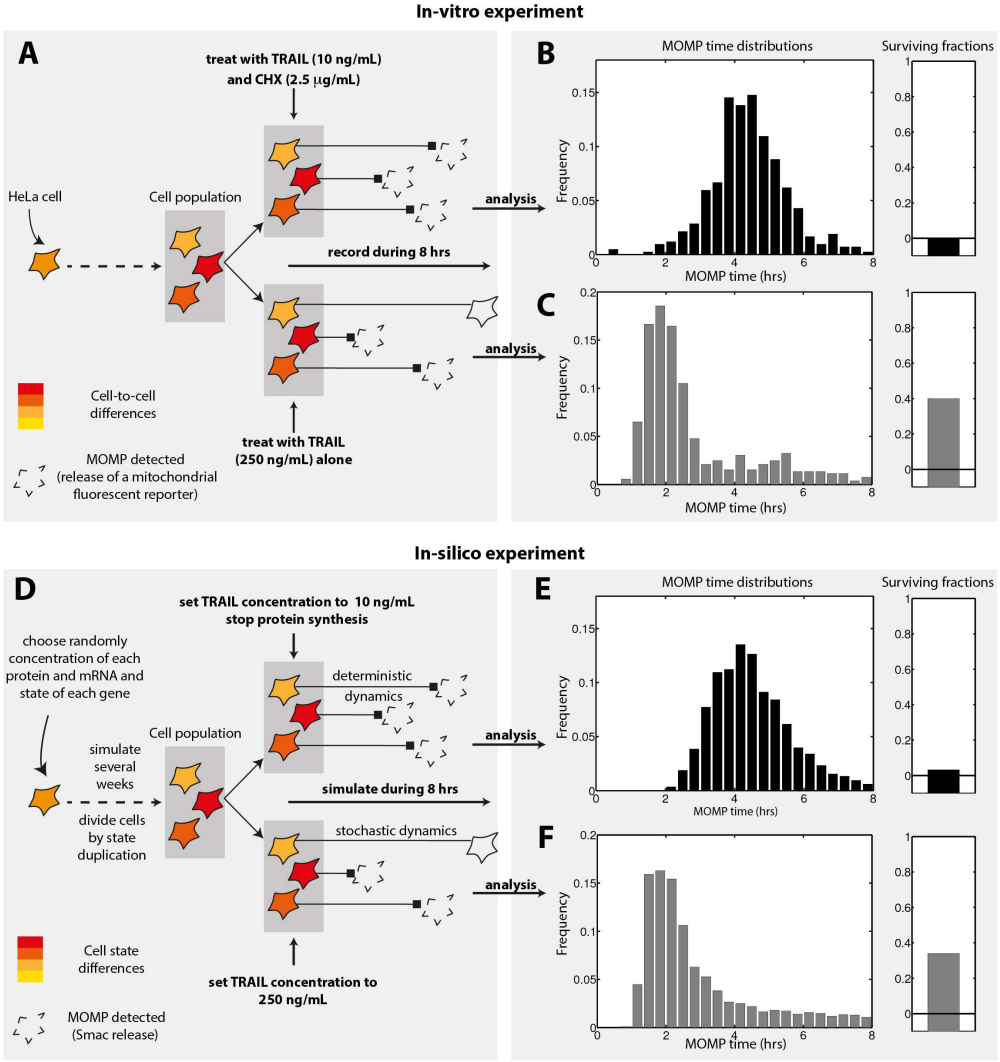

Figure 2. Cell fate variability in TRAIL-induced apoptosis (A-C) Cell fate variability experiments performed by Spencer et al. (2009). (A) HeLa cell populations were treated with either 10 ng/mL of TRAIL and 2.5 μg/mL of cycloheximide (CHX) or 250 ng/mL of TRAIL alone. Cells were tracked during 8 hours by live-cell microscopy and MOMP time was detected via mitochondrial release of a fluorescent reporter. (B-C) Histograms of MOMP times and surviving fractions observed for treatment with (B) TRAIL and CHX (10 ng/mL and 2.5 μg/mL) or (C) TRAIL alone (250 ng/mL). (D-F) In-silico reproduction of those experiments with our model (described in Tables S1-3). (D) Simulations (see Methods for details). (E-F) Results for the (E) TRAIL and CHX or (F) TRAIL alone treatments.

We first asked if the observed cell fate variability could be reproduced. In the model, it is only determined by protein levels at treatment time (behavior is deterministic as synthesis is assumed to be fully blocked by CHX and noise in signaling reactions is neglected), and differences between sister cells are only caused by protein synthesis noise occurring between division and treatment (in agreement with the fact that recently divided sisters died almost

synchronously, we assumed an equal repartition of protein content at division). We found that excellent agreement with observed MOMP time variability can be obtained (Figure 2E). Further analysis revealed that such agreement requires Flip and Mcl1 protein half-life to be short and to fall within a narrow range (between 0.3 and 0.6 hours, Figure S4). This model prediction is consistent with previous measurements in HeLa cells (30 and 40 minutes for Flip short isoform and Mcl1 respectively, Poukkula *et al*, 2005; Nijhawan *et al*, 2003). Oppositely, Flip and Mcl1 mRNA half-life and promoter switching rates are not strongly constrained, probably because their influence on cell fate is limited by the rapid protein level decrease caused by synthesis blockade. We then asked whether our extended model also capture transient cell fate inheritance (Figure 3B). It is the case: fitted models accurately predict the MOMP time correlation between sister cells (Figure 3D - black curve, Figure S5). This non-trivial result shows that the speed at which the sensitivity to TRAIL and CHX fluctuates in single cells is well captured and thus suggests that our generic approach permits to describe fluctuations of protein levels with sufficient accuracy.

Large, rare fluctuations of short-lived anti-apoptotic proteins explain fractional killing and predict transient cell fate inheritance

Spencer and colleagues repeated this experiment but treated cells with TRAIL alone (250 ng/mL). In this condition, an important fraction of cells died fast (MOMP in ~ 2 hours) but 40% were still alive after 8 hours (Figure 2C), illustrating the fractional killing property. Also, cell fate inheritance between sister cells was markedly changed: only young sister cells that underwent MOMP rapidly were importantly correlated (Figure 3B, grey curve). We asked whether the observed cell fate variability, including fractional killing, could be reproduced *in-silico*. Within our modeling assumptions, absence of co-treatment with CHX makes a fundamental difference: as synthesis continues, the effect of gene expression noise during TRAIL-induced apoptosis could be investigated, and comparison with the TRAIL and CHX

condition is insightful. Strikingly, we found that quantitative agreement for both MOMP time distribution and surviving fraction could be obtained (Figure 2F). Robustness analysis showed that rates of the Flip and Mcl1 stochastic protein turnover model, and particularly promoter switching rates, are in this case strongly constrained. Interestingly, MOMP time distribution and surviving fraction constrain those values differently (Figure 4A-B), resulting in an narrow ranges for their values: agreement for both observations together is obtained only when promoter switching rates are both low (Figure 4C). Such low switching rates lead to large, rare fluctuations of protein levels (Figure 4D). Those atypical fluctuations phenotypes are expected to leave a signature at the population level: the shape of the protein level distribution would be bimodal rather than lognormal-like. This property is thus a model prediction.

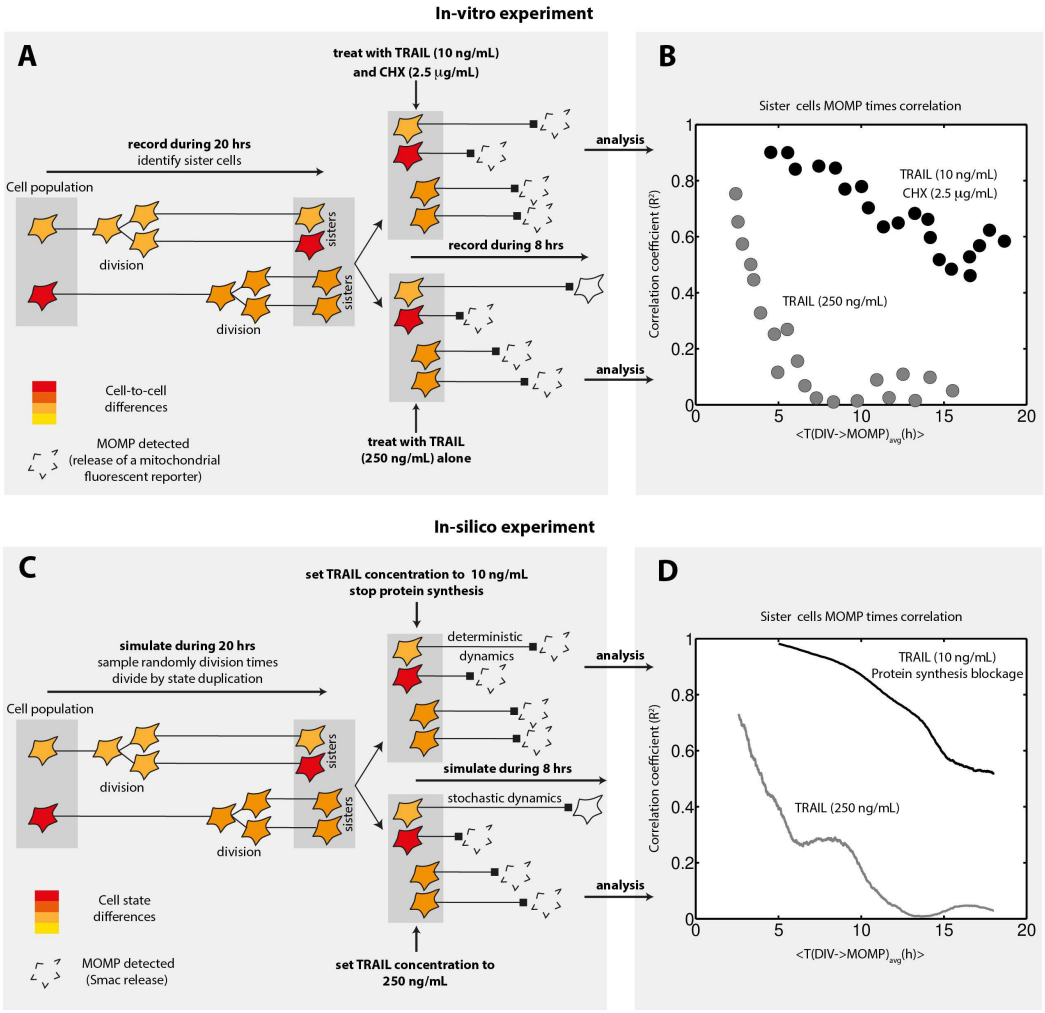

Figure 3. Transient cell fate inheritance in TRAIL-induced apoptosis (A-B) Experiments measuring correlation of MOMP times between sister cells performed by Spencer et al. (2009). (A) HeLa cells were recorded from 20 hours before treatment with TRAIL and CHX (10 ng/mL, 2.5 μg/mL) or TRAIL alone (250 ng/mL). Sister cells were identified to permit comparison of their fate. (B) Quantification of cell fate inheritance was realized by computing the correlation between sister cells MOMP time as a function of the duration between division and MOMP (averaged between sisters). Note that the information content of this experiment is richer than the one described in Figure 2. Sister cells data is presented separately because it contains information about transient memory in cell state, as opposed to the cell fate variability data. (C-D) In-silico reproduction of those experiments with our model. (C) Same rates as in Figure 2D are used. (D) Quantification of cell fate inheritance was applied to simulation results as in (B). See Methods for details.

Those fluctuations are likely to impact how the fate of sister cells diverge with time. Therefore, we asked whether the model could also account for the observed fast loss of cell fate inheritance. Remarkably, the fitted models accurately and robustly predict MOMP time correlations between sister cells (Figures 3D and 4E). Thus, comparison with transient cell fate inheritance data supports that large, rare fluctuations of Flip and Mcl1 could be responsible for the observed cell fate variability.

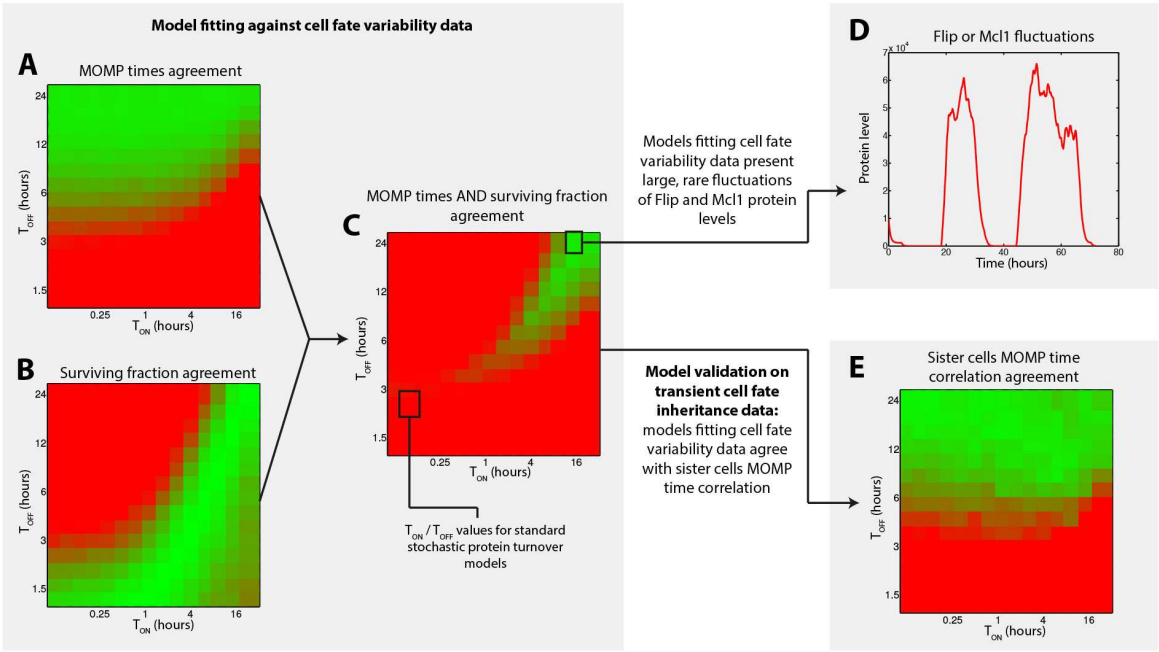

Figure 4. Model fitting to cell fate variability data predicts large, rare fluctuations of Flip & Mcl1 and transient cell fate inheritance. (A-C) Agreement between model prediction and experimental data for (A) death (i.e. MOMP) time distribution, (B) surviving fraction after 8 hours, and (C) both together, for treatment by TRAIL alone (250 ng/mL), as a function of Flip & Mcl1 promoter switching times (other parameters as in Table S1). (D) Representative protein level fluctuations for a Flip & Mcl1 stochastic protein turnover model allowing good agreement for both MOMP time distribution and

surviving fraction. This model has been used for Figures 2,3,5 and 6. All models fitting the data present large and rare fluctuations of Flip and Mcl1 levels. (E) Model-data agreement for MOMP time correlation between sister cells. For (A),(B),(C) and (E), agreement quality increases from red to green, quantification algorithm is detailed in Supplementary Information.

In summary, a refined model of TRAIL-induced apoptosis in which stable proteins are equipped with standard stochastic protein turnover models whereas particularly short-lived proteins Flip and Mcl1 are assigned specific stochastic protein turnover models involving large, rare fluctuations of protein levels is able to quantitatively explain the cell fate variability, including fractional killing, observed after treatment with either TRAIL and CHX or TRAIL alone (Spencer *et al*, 2009). Remarkably, our model does not include any TRAIL induced regulation and therefore these results challenge the generally accepted role of surviving pathway for cell survival during apoptosis (Falschlehner *et al*, 2007). Nevertheless, our results are consistent with previous observations that blockade of NF-κB or Akt pathway in wild type HeLa cells does not change the surviving cell fraction after TRAIL treatment (Braeuer *et al*, 2006; Lalaoui *et al*, 2011). Our well-constrained model also robustly predicts other observations characterizing the transient memory in cell state, i.e. transient cell fate inheritance between sister cells (Spencer *et al*, 2009). Thus, while fitting was performed against data that covered only 8 hours starting from TRAIL application, the temporal scope of the model spans up to 20 hours before TRAIL treatment (most ancient division of sister cells registered).

Accounting for stochastic protein turnover predicts reversible resistance

Recently, reversible resistance was observed among a variety of cell lines (Flusberg *et al*, 2013). Cell populations were submitted to two consecutive TRAIL treatments. The duration between treatments was varied from 1 day to 1 week (Figure 5A), allowing the comparison of TRAIL sensitivity between ‘naïve’, ‘recent survivors’ and ‘ancient survivors’ populations. One-day survivors were significantly more resistant than the initial population, but such

resistance was significantly decreased or even lost in one-week survivors. Thus, cells surviving a first TRAIL treatment are transiently resistant to TRAIL.

To investigate possible origins of reversible resistance, we reproduced those experiments *in-silico* (Figure 5B). Remarkably, we found that our model predicts reversible resistance (Figure 5C): one-day survivors exhibit a significant resistance to a second TRAIL treatment, which disappears after 3 to 5 days. Moreover, resistance in survivors increases with TRAIL dose. Those results demonstrate that stochastic protein turnover alone in TRAIL-induced apoptosis is sufficient to explain reversible resistance. Consistently, blockade of the NF-κB pathway was observed to be unable to change resistance acquisition after TRAIL treatment (Flusberg *et al*, 2013, in MCF10A cells, HeLa cells were not tested). Thus, in addition to explaining fractional killing, accounting for stochastic protein turnover in the TRAIL-induced apoptosis pathway did not only extend the model scope dozens of hours before treatment, but also several days after.

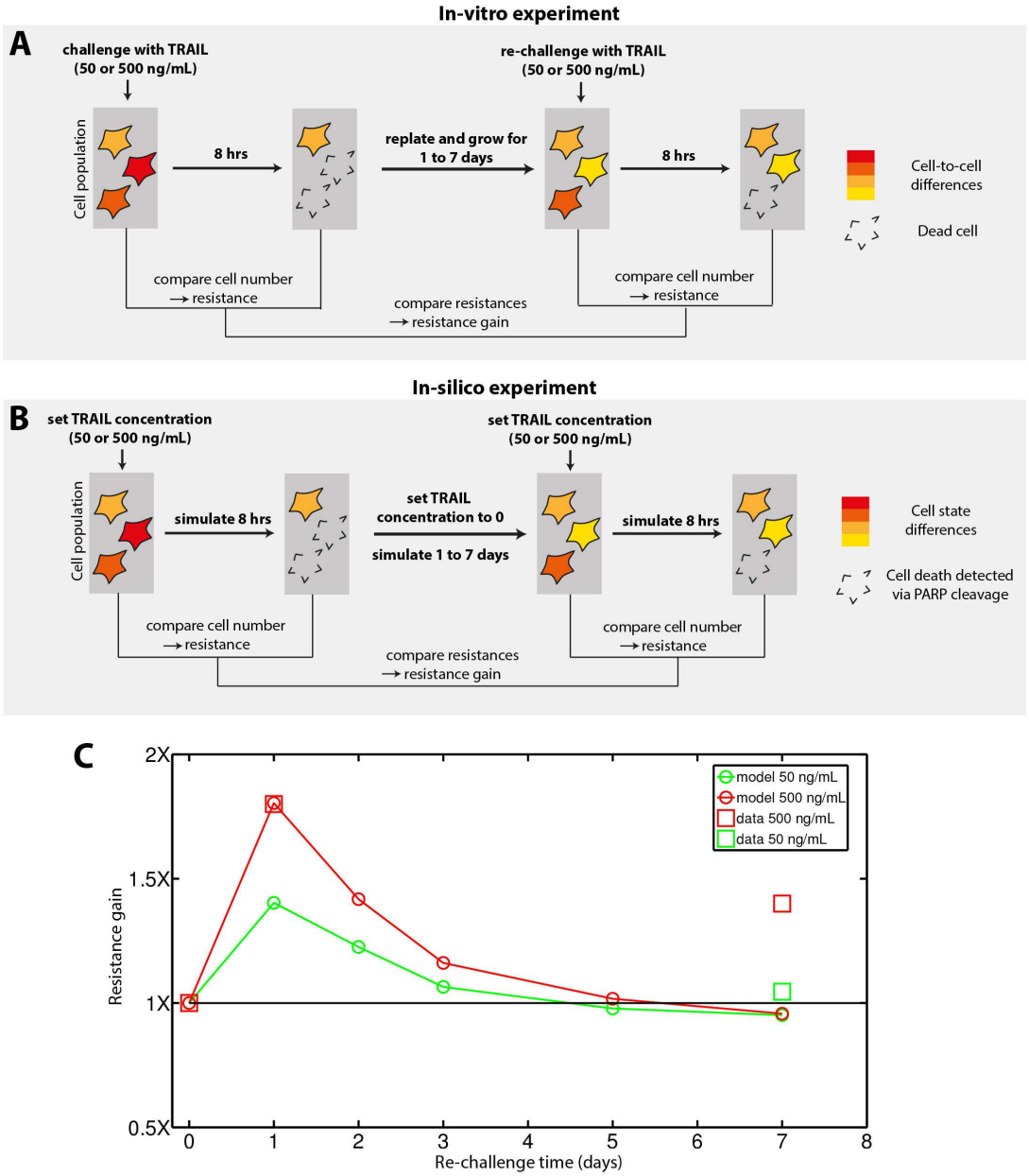

Figure 5. Reversible resistance in repeated TRAIL treatments (A) Schematic description of the 'repeated TRAIL' experiments performed to characterize reversible resistance in HeLa cells (Flusberg et al. 2013, Flusberg & Sorger 2013). (B) In-silico reproduction of these experiments with our model (details in Methods). (C) Resistance gains in surviving cells relative to naïve cells as a function of time between the two TRAIL treatments. Data are shown for experimental observations (Flusberg et al. 2013, Flusberg & Sorger 2013) and model predictions (our study). Direct comparison of resistance gain experimental measurements between the two TRAIL doses (500 and 50 ng/mL, Flusberg et al. 2013 and Flusberg & Sorger 2013 respectively) should be done with care, as the measurement and quantification method differed.

Molecular determinants of fractional killing and reversible resistance

What are the mechanisms behind cell escape to TRAIL-induced apoptosis, either on the short-term (fractional killing) or the long-term (reversible resistance)? Taking advantage of the fact that *in-silico*, all protein, mRNA levels as well as gene activity states can be monitored in single cells, we investigated those questions at the molecular level.

First, we studied the influence on cell fate of pre-existing differences between cells. We compared proteins levels at the time of stimulation between 'future survivors' and the whole population (Figure 6A-B). If the initial level of a given protein contributes in determining cell fate, we expect to find it in higher or lower amount in future survivors. Simulations showed that future survivors strongly stood out by their Mcl1 levels: they had on average twice more Mcl1 proteins than the whole population (Figure 6B, blue bars). Flip also appeared to play an important role in determining cell decision, with 25% more proteins found in future survivors. Smaller but significant effect was also seen for Bid, Bax, Bcl2 and XIAP. The specific role of Mcl1 was confirmed at the gene activity level (Figure 6B, red bars): the fraction of future survivors that were initially in the transcriptionally active state was 2.3 times higher than in the whole population. Although it appears to be a good predictor of cell fate, initial Mcl1 gene activity status does not completely determine survival: neither all Mcl1 "ON" cells survived nor all Mcl1 "OFF" cells died. Thus, if pre-existing differences in protein levels and promoter activities are major determinant of cell fate, stochastic events in gene expression occurring during signal transduction also play a role. While timing of death for cells treated with TRAIL and CHX appeared to be multi-factorial (Spencer *et al*, 2009), our results suggest that cell survival is strongly determined by Mcl1 alone, either through initial protein level or transcriptional activity: only cells able to maintain high levels of Mcl1 long enough would be able to block the triggering of MOMP. Indeed, we found that assuming large and rare fluctuations for Mcl1 alone is sufficient to quantitatively explain cell fate variability, transient cell fate inheritance and reversible resistance (Figure S6).

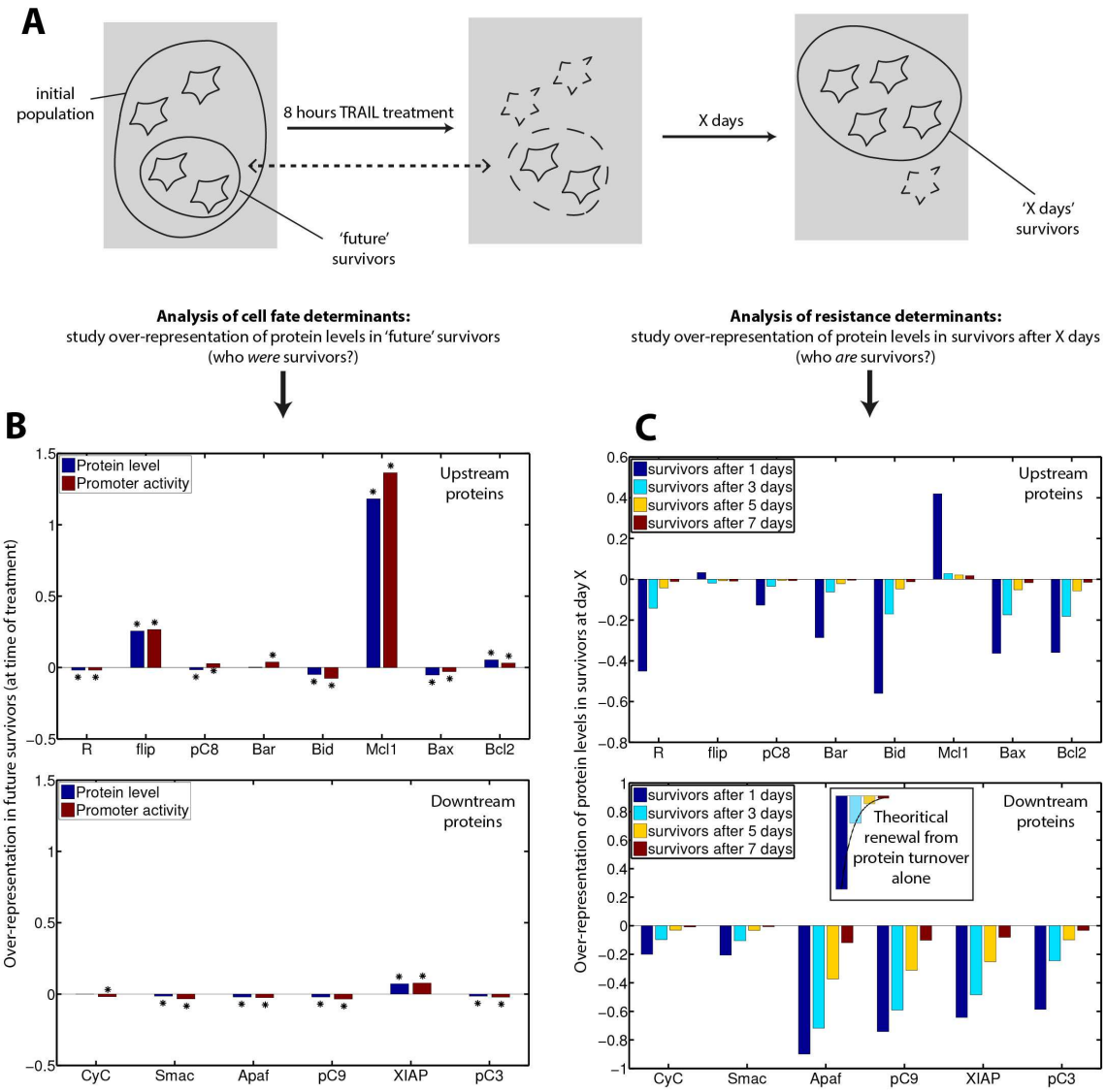

Figure 6. Molecular determinants of cell fate and resistance to repeated TRAIL treatments (A) Cartoon illustrating that the determinants of cell fate and resistance can be studied by analyzing the over-representation of protein levels in 'future survivors' (cells that will still be alive after treatment) at the time of treatment, and in surviving cells at day X, respectively. (B) Cell fate determinants analysis: over-representation (compared to initial population) of protein level (blue) and promoter activity (red) at the time of treatment in 'future survivors'. Asterisks mark differences that passed a 5% significance test (Kolmogorov-Smirnoff for protein levels, Chi-2 for promoter states). (C) Resistance determinants analysis: over-representation of protein levels in surviving cells at day X. Inset illustrates the recovery kinetics expected from protein turnover only (i.e. in absence of significant selection effect or residual signaling activity). Therefore, deviation from such kinetics indicates the presence of a selection effect or residual signaling activity.

Although such dominant control of cell survival by Mcl1 was unexpected, important differences in Mcl1 levels were observed to have a major impact on cell fate in HeLa cells:

Mcl1 over-expression strongly reduces TRAIL-induced apoptosis while silencing via RNA interference strongly sensitize cells (Clohessy *et al*, 2006).

To investigate the determinants of reversible resistance, we then tracked the temporal evolution of protein levels in surviving cells (Figure 6 A&C). Three effects of different nature could be thought to impact those levels. First, selection of cells depending on their protein content should be at play: based on previous results, we expect to see higher Mcl1 levels in survivors. Because of memory effects, promoter activity state and mRNA levels are also expected to be indirectly selected: cells with high protein levels are likely to have high mRNA levels and a transcriptionally active promoter. Those differences should disappear with time because of stochastic fluctuations. Second, all proteins are involved in signal transduction reactions and are therefore potentially used along the process, especially because active forms have a shorter half-life. With time, protein synthesis would restore the initial balance with degradation and thus initial protein levels. Third, there might be residual protein-protein reactions still active long after TRAIL stimulation, resulting in a continued activation of native proteins and therefore delaying the complete reconstruction of initial levels.

Our simulations revealed that all those effects are simultaneously at play, but with a relative importance that depends on each protein specific properties and position in the pathway (Figure 6C). When recovery of protein levels is mostly mediated by protein turnover (in absence of significant selection or continued consumption), it is expected to follow exponential kinetics governed by the turnover rate (Figure 6C, inset). Here, it appeared to be the case only for the death receptor (R), pro-caspase 8, Bar and Bid. The two short-lived proteins Flip and Mcl1 are expected to return to normal levels within a few hours in absence of selection. Instead, Mcl1 levels are still strongly higher than in naive cells one day after TRAIL treatment, consistently with previous results showing that it is strongly selected. This is not the case for Flip, which is less selected and therefore recovers normal levels faster.

Finally, Apaf, pro-caspase 9 and XIAP, which are almost not subjected to selection, exhibit unusual recovery kinetics, incompatible with renewal by protein turnover only: protein levels almost do not change between one and three days after TRAIL exposure, a signature for persisting protein-protein reactions involving the consumption of those proteins. Indeed, detailed analysis of single cell trajectories showed that cytochrome c, in limited amount but sufficient to catalyze Apaf activation, could be slowly released from mitochondria in survivors, even long after TRAIL treatment.

Together, those results indicate that recovery phenotypes in surviving cells result from a combination of three distinct effects: selection during apoptosis, transcriptional noise as a driving force tending to reset protein levels to their initial, pre-stimulus distribution, and long-term residual signaling activity. This non-trivial interplay explains why it is difficult to understand the recovery process and justifies the use of modeling. As opposed to the death process, which involves a sharp and complete activation of effector caspases, our results suggest that recovery in cells that did not commit to death is a slow and complex process.

Discussion

Spencer *et al* (2009) made two particularly insightful observations about TRAIL-induced apoptosis. First, recently born sister cells died almost synchronously when treated with TRAIL and a protein synthesis inhibitor, while in contrast, unrelated cells died after highly variable durations. This demonstrated that TRAIL signaling is mostly deterministic when protein synthesis is blocked and that the timing of death is determined by the cell internal state at the moment of treatment. Second, they observed that such synchrony in sister cells death is gradually lost as their age (time between division and treatment) increases. This showed that the cell 'TRAIL sensitivity state', i.e. the part of cell internal state involved in death timing determination, naturally fluctuates over a dozen of hours. In addition, their modeling work highly suggested that such 'TRAIL sensitivity state' is mainly composed of the

various levels of the proteins acting in the extrinsic apoptosis pathway. In parallel, important progress on the characterization of the stochasticity in gene expression has been made: the two-state transcriptional bursting model was shown to be realistically accurate and several approaches to infer its parameters were proposed, thus enabling the quantitative modeling of protein fluctuations in single cells (Dar *et al*, 2012; Paszek, 2007; Raj *et al*, 2006; Suter *et al*, 2011; Viñuelas *et al*, 2013).

Modeling protein fluctuations in TRAIL-induced apoptosis

In this study, we merged those two approaches by integrating such stochastic models of gene expression within an existing kinetic model of TRAIL-induced apoptosis (Albeck *et al*, 2008). Doing so provides advantages compared to previous approaches to account for cell-to-cell variability in protein levels (Spencer *et al*, 2009; Gaudet *et al*, 2012). First, variability is not considered as an "input" parameter but arises naturally from stochastic fluctuations. Therefore, the dynamics of this variability is intrinsically represented, allowing investigating the effects of transient memory in protein levels. Second, the influence of protein synthesis noise during TRAIL-induced apoptosis could also be investigated. Importantly, we followed a parsimonious parameterization strategy, motivated by the fact that fluctuations of short-lived proteins are more sensitive to the precise kinetics of transcriptional bursting than long-lived proteins. Thus, a majority of proteins were equipped with standard stochastic protein turnover models, and only the short half-lived anti-apoptotic proteins Flip and Mcl1 were given particular attention.

The sister cells experiment for which cells were treated with TRAIL and CHX provided ideal data to validate our modeling approach: in that case, behavior is mostly deterministic as soon as treatment starts and therefore only fluctuations occurring before treatment are responsible for death time variability and de-correlation between sister cells. Because our model was able to quantitatively reproduce the MOMP time distribution and then accurately predicted sister

cells correlation, our modeling approach, which is generic, appears as a promising tool to investigate the effect of protein fluctuations on signal transduction.

Also, it is worth noting that the transposition of fluctuation timescales from individual proteins into the "TRAIL sensitivity state" is not trivial: while in our model, stable proteins levels are mixed in about 40 hours, cells were switching between 'fast dying' and 'slow dying' states more rapidly (about 10-15 hours). Apparently, some combinatorial effect is at play, justifying the need of mechanistic models of protein-proteins reactions to link protein-level timescales with more high-level "phenotypic transitions" such as the ones characterized by Gupta *et al* (2011).

Questioning the role of survival pathways

Several studies reported that TRAIL can induce survival pathways (Chaudhary *et al*, 1997; Son *et al*, 2010; Sun *et al*, 2011). How such induced changes in protein synthesis could affect signal transduction and eventually stop apoptotic signaling remains unclear. In particular, how would the induction of survival pathway result in the survival of a fraction of the cell population? In fact, a simpler question should be addressed first: what is the contribution of constitutive protein synthesis noise, which is responsible for pre-existing differences between cells, on cell fate variability? With our model, it was possible to investigate this question by comparing results between the TRAIL and CHX and TRAIL alone treatments.

A striking result of our study is that fractional killing could be quantitatively explained without assuming any TRAIL-induced regulation. Instead, it is sufficient to assume a constitutive heterogeneity in the levels of short-lived anti-apoptotic proteins Mcl1 and Flip, caused by rare switches between long periods of gene activity or inactivity. Importantly, this explanation is supported by the fact that the observed rapid loss of MOMP time correlation between sister cells was accurately predicted.

While they challenge current opinion on the role of survival pathways in TRAIL-induced apoptosis, those results are consistent with observations made on wild type HeLa cells that neither blocking NF-κB response nor inhibiting the Akt pathway do significantly change the surviving cell fraction after TRAIL treatment (Braeuer *et al*, 2006; Lalaoui *et al*, 2011). Interestingly, as in our model, a pivotal role for Mcl1 and Flip has been reported for TRAIL-induced apoptosis in HeLa cells (Clohessy *et al*, 2006; Lemke *et al*, 2013). Our model provides a simple and minimalistic explanation for Mcl1 and Flip fluctuations: constitutive expression with rare switches between transcriptionally active and inactive states. However, both proteins are known to be regulated at many levels (Shirley & Micheau, 2013; Shore & Warr, 2008). Therefore, we cannot exclude other mechanisms, provided that they generate similar fluctuations. In particular, Lemke and colleagues (2013) recently showed that transcription of Mcl1 and Flip (but not other apoptotic proteins) could be dramatically reduced by selective inhibition of the CDK9 kinase, known to be involved in transcriptional elongation. Spontaneous fluctuations in CDK9 activity could therefore be a possible cause for the important constitutive heterogeneity in Mcl1 and Flip levels predicted by our model. Importantly, one should search for fluctuation mechanisms that do not depend on TRAIL stimulation.

Origins of reversible resistance: joint effect of selection and stochastic protein turnover

A second significant result reported here is that our model predicts reversible resistance, showing that constitutively noisy protein synthesis, protein-protein interactions and protein degradation are by themselves sufficient to explain a dose-dependent, significant increase of resistance in recent survivors and its gradual loss within 3-5 days. *In-silico* analysis at the molecular level revealed that reversible resistance was shaped by a complex interplay between 1) selection based on protein levels and transcriptional activity, 2) protein turnover and 3) residual signaling activity.

Comparing our results with observations on MCF10A cells (Flusberg *et al*, 2013) suggests that reversible resistance is cell-line specific, as in MCF10A cells, it seems to involve preferentially Flip and DISC assembly rather than Mcl1 and Bid. Alternately, it could be that the role of Flip is under-estimated in our model because DISC assembly involves several steps and actors that are not represented in EARM. Thus, extending EARM with a more mechanistic representation of DISC assembly might help to further precise the relative contribution of Flip and Mcl1 in cell survival.

Limitations

The modeling approach applied here suffers from several limitations. First, we considered only one gene copy per native protein modeled in EARM, thus neglecting the effect of multiple copies. Functionally redundant proteins (e.g., caspases 3 and 7) and splice variants (e.g., c-Flip isoforms) were also aggregated under a single species. Second, we assumed that the stochastic events affecting protein expression are all mutually independent. This apparently reasonable hypothesis cannot account for observations that in some cases, the levels of different proteins appear significantly correlated (Sigal *et al*, 2006; Gaudet *et al*, 2012). Such correlated expressions could originate from common transcription factors or coordinated chromatin-state transitions (Ozonov & van Nimwegen, 2013).

Accounting for gene expression noise appears necessary to investigate signal transduction

In this work we focused on TRAIL-induced apoptosis, but our modeling approach could be applied to the study of other signal transduction pathways. Our results showed that even in absence of induced gene regulation, gene expression noise interacts with signaling dynamics on a non-trivial manner. Thus, even in contexts where the influence of induced gene-regulation is indisputable, its sound quantification probably requires to investigate first the role of constitutive gene expression noise. Only then models could be enriched parsimoniously with well-characterized regulatory links until all observations are successfully

explained. Significant advances to allow such detailed characterization of gene regulation occurred recently (Dar *et al*, 2012; Molina *et al*, 2013; Neuert *et al*, 2013; Tay *et al*, 2010). Following such approaches could significantly extend the reach of models of signal transduction towards accurate, single-cell level description of populations submitted to varying signaling contexts over multiple cell generations.

Material and Methods

Kinetic Model of TRAIL-induced Apoptosis

We used the EARM model described in Spencer *et al* (2009) to represent protein-protein reactions. No kinetic rates were changed, with the exception of the rate of pC6 cleavage by C3, which was set to zero to remove the feedback loop. Degradation rates of non-native forms were modified according to biological knowledge (Table S3). The effect of those changes is discussed in Supplemental Information.

Building Stochastic Protein Turnover Models

Rates of the standard stochastic protein turnover models and rates of Flip&Mcl1 models were determined following an algorithm constructed to incorporate constraints based on biological knowledge (Figure S4). Their values are given in Table S1-2.

In-silico Sister Cells Experiment

The simulation procedure is detailed in Supplemental Information. Briefly, mother cell states (promoter activity, mRNA and protein levels) were chosen by Monte-Carlo sampling and the two sister cells were constructed by duplication of the mother cell state. Promoter activity and mRNA fluctuations were simulated using an implementation of the Gillespie algorithm, for the ODEs governing evolution of all protein levels, the Semi-Implicit Extrapolation method implemented in C++ was used (*Numerical Recipes*). MOMP was considered to have occurred when half of mitochondrial Smac has been released. For each protein turnover model of Flip

& Mcl1 explored, 10^4 pairs of sister cells were simulated (10^5 for data used in Figures 2, 3, 5 and 6).

In-silico Repeated TRAIL Experiment

Detailed description is given in Supplemental Information. Briefly, a naïve population of 10^4 cells was obtained as in the sister cells experiment. Cells divided after a cell cycle duration normally distributed around 27 hours (3 hours standard deviation). Death was detected via cPARP levels as in Gaudet *et al* (2012).

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