

Parole terms for a killer

Directing caspase3/CAD induced DNA strand breaks to coordinate changes in gene expression

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In a series of discoveries over the preceding decade, a number of laboratories have unequivocally established that apoptotic proteins and pathways are well conserved cell fate determinants, which act independent of a cell death response. Within this context, the role for apoptotic proteins in the induction of cell differentiation has been widely documented. Despite these discoveries, little information has been forthcoming regarding a conserved mechanism by which apoptotic proteins achieve this non-death outcome. In the following discussion, we will explore the premise that the penultimate step in apoptosis, genome wide DNA damage/strand breaks act as a conserved genomic reprogramming event necessary for cell differentiation.⁵ Moreover, we hypothesize that directed DNA damage, as mediated by known apoptotic proteins, may participate in numerous forms of regulated gene expression.

Introduction

DNA damage is largely assumed to be a detrimental event and is frequently associated with impaired cell survival. In addition, DNA damage is a common molecular trigger for the development of oncogenic mutations. For example DNA damage/strand breaks precede a recombination of two distinct genetic loci to produce a hybrid gene with growth altering properties. Given the dire outcomes associated with DNA damage, the cell has evolved a number of DNA repair pathways which

recognize and redact genome damage that occurs from a variety of external insults.

Despite the propensity to consider DNA damage as a solely negative phenomenon, a growing body of evidence suggests that focal DNA damage is in fact required for normal cell function. Specifically, controlled or non-random DNA damage appears to be a conserved mechanism which propagates alterations in gene expression. The best studied example in this regard is the regulated DNA breakage that propels adaptive immunity. Here, the Rag1/2 nuclease complex induces controlled DNA breaks at specific loci that are paired with variable recombination events to create the unique genes that underwrite the diversity of antibody production.¹ Another well documented example of tolerable or beneficial DNA breakage occurs during the exchange of genetic material between homologous chromosomes in meiotic crossover. In this instance, DNA breaks are induced by the topoisomerase II-like protein Spo11, which propels an exchange between large intergenic regions.²

Additional studies suggest that directed DNA strand breaks may promote gene expression independent of follow-on recombination events. For example, work from the Rosenfeld Laboratory has shown that glucocorticoid induced gene expression is dependent on a topoisomerase IIbeta mediated DNA strand break. The strand breaks are directed to the promoter region of the glucocorticoid responsive gene, an alteration that prompts histone modifications which are favourable to initiating

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gene transcription.^{3,4} Undoubtedly, there is an inherent risk associated with such DNA damage yet these observations establish that limited DNA strand breaks can yield a notable beneficial outcome for a cell.⁴

Recently, work from our laboratory has transformed the paradigm described above and shown that genome wide DNA strand breaks act as a key regulatory step to promote muscle cell differentiation.⁵ The DNA strand breaks are formed by a transient activation of caspase activated DNase or CAD, an observation that suggests apoptotic proteins and pathways act as conserved genomic reprogramming factors.

Caspase 3/CAD Signaling: The Executioners

The caspase family of cysteine proteases signal through proteolytic cleavage, altering the activity of an extensive repertoire of substrates. Caspase 3 is an integration point for a variety of canonical cell death pathways, acting to target cytosolic and nuclear factors which in turn accelerate cellular demise.⁶ One key feature of this potent signal is the ability to activate the primary nuclease involved in disseminating extensive DNA strand breaks, termed caspase activated DNase or CAD.⁷ DNA damage in the form of genomic fragmentation is a well characterized component of cell death or apoptosis. Although early stage DNA fragmentation is not an absolute requirement for all early stage forms of apoptotic cell death, these nuclear events are assumed to improve the efficiency of the process.⁸ Indeed, the release of nucleotides and presentation of nucleosomes at the cell surface promote the removal of degenerate cells through attraction of phagocytic cells.^{9,10}

The principle regulation of CAD comes by formation of a restrictive complex with its inhibitor, ICAD, an interaction that physically restrains inactive CAD monomers from dimerizing into the active form. Two isoforms of ICAD are observed, ICAD-long (L) and ICAD-short (S), with the ICAD-L isoform having the most defined role in regulating CAD. Association by N-terminal CIDE domains begins when the CAD

protein is synthesized, here ICAD-L acts as a specific chaperone along with Hsp70 and Hsp40 to properly fold CAD.^{11,12} Following translation the ICAD-L/CAD complex localizes to the nucleus, where it remains highly mobile; however, stable interactions with DNA have been noted suggesting that CAD can be activated not only in the free nuclear space but also in a DNA associated complex.¹³⁻¹⁵

To activate CAD, caspase 3 targets ICAD for proteolytic cleavage at 2 aspartic acids, D117 and D224. This caspase directed cleavage destabilizes the ICAD/CAD interaction and allows CAD dimerization. The CAD dimer aptly resembles an open scissor like structure, with the catalytic site of the dimer located in a crevice structure large enough to accommodate double stranded DNA.¹⁶ The substrate DNA fully enters this structure and a double stranded DNA break is catalyzed. This protein conformation is believed to exclude nucleosome associated DNA, localizing the DNA break between nucleosomes. Under apoptotic conditions the extent of CAD activation is such that periodic DNA laddering is observed.⁸

Caspase 3/CAD Mediated DNA Strand Breaks: A Vital Genome Reprogramming Event

In addition to a well characterized role in apoptosis, caspase 3 activation is also a highly conserved step in the induction of cell differentiation. Transient caspase 3 activity has been shown to be essential for differentiation of most somatic cell types studied and for the maturation of both ES and germs cells.¹⁷ The extent of caspase 3 activation appears to control the balance between differentiation and apoptosis, with lower versus higher levels of activity controlling each cell fate respectively.¹⁸ Defining the caspase 3 substrates that convey the differentiation signal has been of considerable interest, yet limited information has been forthcoming. Studies have suggested that caspase cleavage activation of select kinases propels the differentiation program whereas other studies have shown that cleavage inactivation of transcription factors establishes a permissive environment for the process.¹⁹⁻²³ Although these specific caspase substrate interactions

appear to be critical, these same catalytic events do not explain the ability of caspase 3 to induce the global genome reprogramming that typifies cell differentiation.

Recently, we have reported that caspase 3 activates CAD in healthy muscle cells and that this step is essential for completion of myogenic differentiation.⁵ During early stages of differentiation, myoblast nuclei are subject to CAD dependent DNA strand breaks and inhibition of CAD activity (by limiting caspase 3 activation, repressing CAD expression or overexpressing a non cleavable version of the CAD inhibitor ICAD) leads to a near complete blockade in differentiation with a concurrent loss in the formation of strand breaks. The CAD mediated strand breaks are in part permissive, as the induction of the critical regulatory factor p21 is dependent on a caspase 3/CAD directed strand break within the p21 promoter.

Nevertheless, like many interesting discoveries, our recent work opens the door to many more questions than was answered in the initial study. Paramount among the unresolved mechanisms, once CAD is activated how is the DNase restrained sufficiently to induce strand breaks yet not destroy the genome? We hypothesize that two independent mechanisms may contribute to the restrained activation of CAD. First, we anticipate that it is the DNA associated pool of CAD that is activated during differentiation. CAD has been shown to be complexed with ICAD in a nuclear position, a localization that would permit ready activation once caspase 3 was activated.^{13,14} In contrast, the excessive activation of caspase 3 during apoptosis would allow for targeting of both the DNA bound and unbound CAD/ICAD complexes, the later being free to inflict indiscriminate breaks throughout the genome. Consistent with this hypothesis we have noted that the p21 promoter is subject to consistently localized strand breaks during differentiation, while apoptotic muscle cells display widespread formation of strand breaks that are variable from experiment to experiment (see Fig. 4 in ref. 5). A confirmation of our hypothesis will require a definitive visualization of distinct ICAD/CAD pools and/or a mapping of the CAD targeted genome that is specific to each cell fate

outcome. Second, we hypothesize that caspase targeting of ICAD during differentiation is directed at the D117 cleavage site, whereas the greatly elevated caspase activity associated with apoptosis would result in cleavage at both D117 and D224. In this model of CAD activation, caspase cleavage at the D117 site would lead to a partial release of CAD while caspase targeting of both sites would remove any ICAD mediated inhibition with a higher resulting level of CAD activity. We have noted an ICAD cleavage event during differentiation that is consistent with a D117 only deletion, and a mutation in this site that renders ICAD caspase resistant leads to a reduction in myoblast differentiation (see Fig. 2 in ref. 5).

Our observations demonstrate that caspase 3 activation of CAD (through ICAD cleavage and release of active CAD) is the primary step in promoting DNA damage/strand breaks during myoblast differentiation. Nevertheless, caspase 3 is known to target a wide variety of substrates during apoptosis and cell differentiation.¹⁸ Therefore, we hypothesize that caspase 3 targets multiple proteins to ensure efficient activity of the CAD nuclease. Apoptotic signalling pathways modify chromatin ultra-structure prior to DNA damage and nuclear dissolution. In this regard, post-translational modifications of histones assume prominent regulatory roles. As such it is reasonable to assume that similar events pre-empt or assist CAD to promote DNA damage during cell differentiation. Specifically, we assert that the ste20-like kinase MST1 participates in the formation of caspase mediated nicks/strand breaks in differentiating myoblasts by phosphorylating and modifying the activity of chromatin regulatory proteins. This supposition is based on the following observations. First, MST1 was initially characterized as a pro-apoptotic, caspase 3 sensitive kinase and more recently has been demonstrated to directly phosphorylate histone H2B (at serine 14) leading to chromatin compaction and apoptosis.²⁴⁻²⁶ Secondly, our laboratory was the first to describe a non-apoptotic function for MST1, i.e., MST1 as a pro-differentiation kinase.²² Subsequent to our observations, numerous groups reported a role for MST1 in limiting cell cycle progression in

both mammalian and *Drosophila* systems (reviewed in Zeng and Hong 2008).²⁷ Our experiment was noteworthy in that an activated MST1 kinase (caspase 3 cleavage activated kinase domain) provided a partial rescue of differentiation in caspase 3 null myoblasts, yet prolonged expression of MST1 led to formation of picnotic nuclei and apoptosis.²² Together, these observations suggest that MST1 may stimulate differentiation by modulating or by activating chromatin remodeling protein(s) to enhance CAD activity.

Caspase 3 can also target DNA binding proteins to modulate gene expression and several of these targets have the potential to modify CAD access to sensitive genomic elements. The matrix attachment protein, Special AT-rich binding protein 1 (SATB1), is a global organizer of chromatin that facilitates the interaction of DNA elements termed matrix attachment regions (MARs) to the nuclear matrix. SATB1 is a demonstrated cleavage target of caspase 3/6 under both apoptotic and non-apoptotic conditions.²⁸⁻³⁰ Cleavage of SATB1 releases MARs from the nuclear matrix, leading to chromatin disruption.³¹ A specific role for caspase cleavage of SATB1 during differentiation has not been demonstrated, yet it is reasonable to suggest that removal of SATB1 may in some instances expose genomic elements that are targeted by CAD to coordinate gene expression.

CAD activity during cell differentiation may also be moderated by additional chromatin modifications independent of caspase function. CAD/ICAD association to DNA can be potentiated by association with the C-terminal of histone H1, and this interaction can further stimulate the nuclease activity of CAD.³² Specifically, CAD has been reported to dynamically associate with the histone H1 variants, namely H1.5 and H1.0 in both healthy and apoptotic cells.³³ Moreover, H1 variants can affect specific gene expression by cooperating with additional regulatory factors.³⁴ This preferential association to histone variants could partly influence where in the genome CAD inflicts DNA strand breaks. Of interest, coordination between the H1.5 variant and the transcription factor Msx1 has been demonstrated to occur at the promoters

of repressed genes in proliferating myoblasts.³⁵ For example, expression of the cell cycle inhibitor p21 (which we have identified as a bona fide CAD target) is impeded by Msx1 prior to myoblast differentiation.³⁶ Based on our observations a reasonable conjecture is that CAD may be directed to relieve this repressed state and engage p21 expression by interacting with histone H1.5 and thereby displacing Msx1. How operative this mechanism is and how extensive across the genome it may be will require further investigation.

Curbing the Caspase3/CAD Signal

The transient activity of caspase and CAD in differentiating cells suggests the deployment of a mechanism(s) to moderate or terminate this signal prior to inducing extensive DNA damage. Several studies have examined the signal cascades that activate and modulate caspase 3 during non-death responses, yet a definitive control mechanism has not been elucidated.³⁷⁻³⁹ Such inactivation could indirectly end CAD activity by discontinuing proteolysis of ICAD. Intact ICAD (both long and short isoforms) can disassemble the CAD dimer, moreover our observation that ICAD-L is only partially cleaved (with a large pool of intact ICAD-L remaining) suggests that this mechanism maybe operative to prevent extensive DNA fragmentation.¹⁶

Intriguingly, alternate methods that block the nuclease activity of CAD have been noted. Admittedly, these cellular responses have been implicated primarily in response to caspase activity in apoptotic settings or to restrain CAD activity when this nuclease is activated as a by product of another molecular mechanism. Nevertheless, it is reasonable to suggest that these same factors moderate CAD activity during cell differentiation. Polyribosylation of CAD by PARP-1 can further restrict CAD nuclease activity, and a transient increase in PARP-1 activity is reported in skeletal muscle differentiation concurrent with the observed DNA strand break formation reported in Larsen et al.^{5,40,41} We have not examined a role for PARP-1 in CAD mediated DNA strand breaks and repair, yet PARP-1 has been demonstrated to play a role in regulating

gene expression that results from controlled DNA strand breaks in response to glucocorticoid stimulation. Nerve growth factor (NGF) may also provide a signal conduit to moderate CAD activity in both apoptotic and cell differentiation scenarios. For example, NGF protects neuronal cells from apoptosis through a number of mechanisms, two of these mechanisms involve inhibition of CAD. NGF stimulates nucleophosmin and Ebp1 to directly interact with CAD, diminishing its nuclease activity while not preventing caspase cleavage of ICAD itself.^{42,43} Given that caspase 3 has been shown to mediate neuronal differentiation, a valid interpretation of the above experiment may be that NGF restrains CAD activity to act as a moderating influence on gene expression during neuron differentiation (as in skeletal muscle cells) while limiting a global escalation of CAD activity.²¹

CAD Induced DNA Strand Breaks as a General Component of Gene Expression

An intriguing question that arises from the study of Larsen et al. is whether CAD induced DNA strand breaks are simply a differentiation specific regulatory event or is CAD directed DNA damage a common mechanism for altering gene expression?

As noted above, the role of DNA strand breaks regulating changes in gene expression and genomic reprogramming has been characterized in a number of processes. However, in many instances the induction of the DNA breaks have been linked to proteins with weak or questionable nuclease activity. As such it is reasonable to assume that localized CAD activation may coordinate with the other DNA damage related proteins to promote break induced alterations in gene regulation. Glucocorticoid stimulated gene expression involves topoisomerase II dependent DNA strand breaks. These breaks are directed by the estrogen receptor α (ER α) and stimulate target gene expression through PARP-1 mediated exchange of histone H1 for HMGB1/2.^{3,44} A cooperation between CAD and topoisomerase II in stimulating the formation of glucocorticoid stimulated breaks was not examined in

this study, yet such a coordination could effectively direct and stimulate the induction of DNA strand breaks. Indeed, an interplay between CAD and topoisomerase II has been reported in the initial induction of DNA strand breaks during apoptosis, and the proteins co-localize in both healthy and apoptotic nuclei.⁴⁵ These observations combined with weak inherent nuclease activity of topoisomerase II suggest that CAD may provide the operative strand break capacity in such forms of gene regulation.

DNA demethylation has been postulated to involve the formation of DNA strand breaks as a means to excise the methylated base followed by a DNA repair step. In this regard several proteins have been implicated that can also contribute to the formation of regulated DNA strand breaks. The cytidine deaminase AID has been reported to act as a demethylation agent in primordial germ cells and in the nuclear reprogramming of somatic cells via induced pluripotency (iPS).^{46,47} AID has been well studied for its role in antibody diversification where it is thought to stimulate a directed DNA strand break through deamination leading to activation of the base excision repair pathway.⁴⁸ AID has been shown to associate with methylated promoters of pluripotency genes in fibroblasts but not unmethylated promoters in undifferentiated ES cells, and knock-down of AID gene expression impeded the demethylation of these promoters in heterokaryon fusions.⁴⁷ Interestingly global DNA demethylation is observed in skeletal muscle differentiation concurrent to the period of CAD induced DNA strand breaks.⁴⁹ Although we observed unaltered DNA methylation at the p21 promoter, these breaks may signal at other loci for the removal of the repressive mark, a step that may be dependent on a CAD like excision event. Alternatively, given the role of caspase/CAD in driving cell fate outcomes that are directly antagonistic to iPS (i.e., cell differentiation) achieving effective reprogramming may require a direct inhibition or limitation of caspase/CAD activity. As caspase mediated signal events are broadly conserved cell fate determinants, exploring the impact of a caspase/CAD signal nexus during nuclear reprogramming should be a priority.

Determining the genomic targets of caspase 3 activated CAD will assist in developing appreciation for the role these DNA strand breaks play in regulating gene expression (Fig. 1). Mapping these sites using rapidly developing next generation sequencing technology and determining protein interactions that direct CAD will begin to establish this understanding. Further examination of epigenetic changes that occur at these sites will provide insight into how the strand break is utilized to regulate gene expression not only in differentiation but potentially in other genomic reprogramming events.

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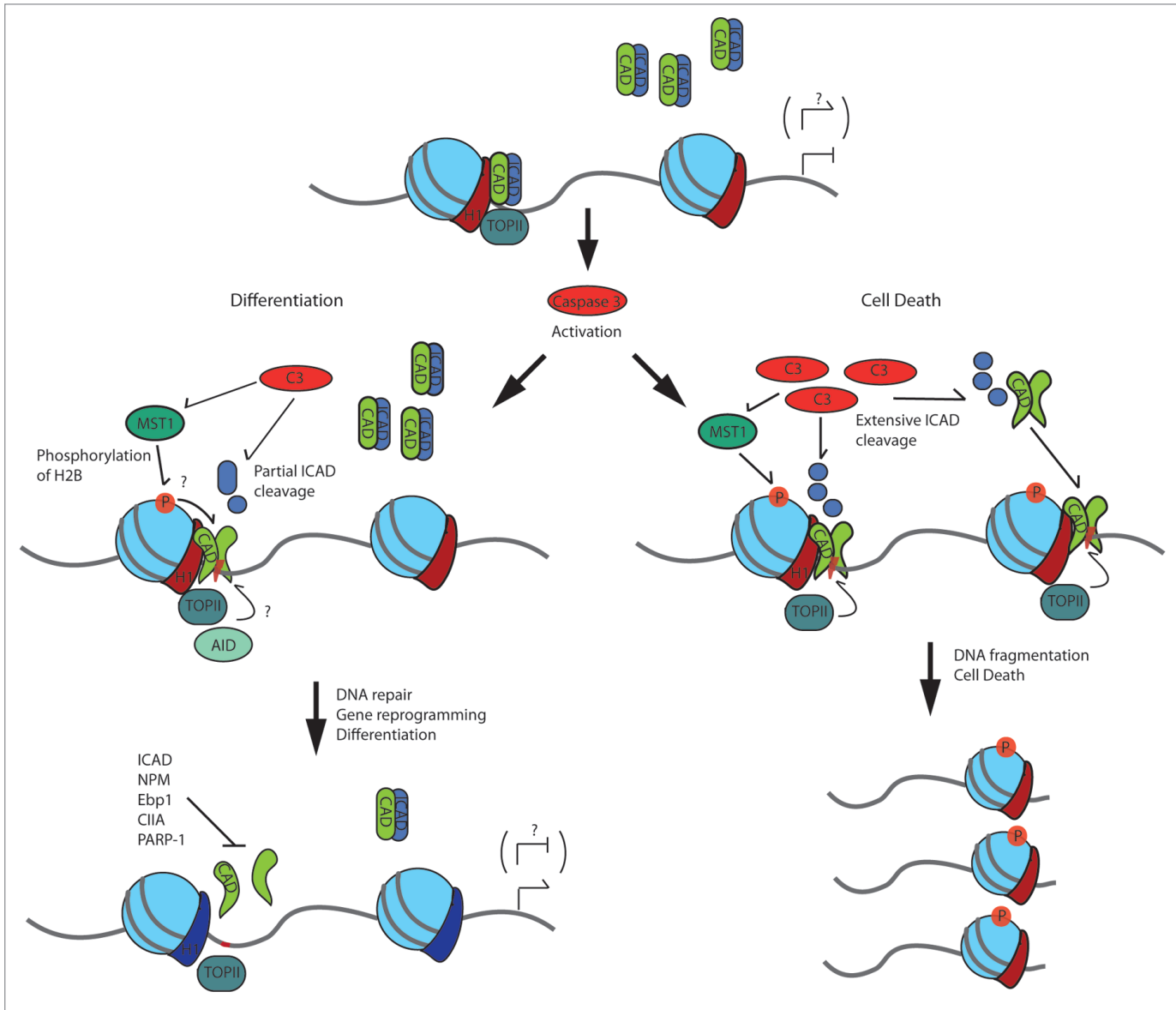


Figure 1. Caspase 3 activation balances the dissemination of CAD induced DNA strand breaks for gene reprogramming or fragmentation. This model depicts several factors that may regulate CAD induced DNA strand breaks in physiologic and apoptotic conditions.

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