Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks

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Caspase 3 is required for the differentiation of a wide variety of cell types, yet it remains unclear how this apoptotic protein could promote such a cell-fate decision. Caspase signals often result in the activation of the specific nuclease caspase-activated DNase (CAD), suggesting that cell differentiation may be dependent on a CAD-mediated modification in chromatin structure. In this study, we have investigated if caspase 3/CAD plays a role in initiating the DNA strand breaks that are known to occur during the terminal differentiation of skeletal muscle cells. Here, we show that inhibition of caspase 3 or reduction of CAD expression leads to a dramatic loss of strand-break formation and a block in the myogenic program. Caspase-dependent induction of differentiation results in CAD targeting of the p21 promoter, and loss of caspase 3 or CAD leads to a significant down-regulation in p21 expression. These results show that caspase 3/CAD promotes cell differentiation by directly modifying the DNA/nuclear microenvironment, which enhances the expression of critical regulatory genes.

development | gene expression | non-apoptotic caspase activity | epigenetics

Differentiation is a deterministic process that ensures cell specialization in complex organisms. In general terms, the cellular diversity that originates from differentiation has been attributed to the activation of lineage-specific transcription factors (1, 2). Despite the propensity to consider differentiation as a tissue-specific event, emerging evidence suggests that this cell-fate choice may also be dependent on the activity of a common signal pathway or protein. One factor that seems to be highly conserved in the induction of differentiation is the caspase 3 protease (3). Caspase 3 was originally identified as the penultimate step in apoptotic/programmed cell-death pathways, acting to cleave vital protein substrates through aspartic acid-directed targeting (4). Subsequently, transient caspase activation has been reported to be indispensible for the maturation of numerous cell lineages across a broad range of species (3, 4).

The mechanism by which caspase 3 promotes nondeath outcomes remains poorly defined. A number of studies have shown that differentiation is dependent on caspase cleavage of select kinase substrates (5–7). For example, caspase 3 induction of skeletal myoblast differentiation is associated with the cleavage activation of the ste-20–like kinase MST1. However, restoration of active MST1 in caspase null myoblasts provides only a partial rescue of the differentiation defect, indicating that caspase activity must engage additional substrates to promote or sustain nondeath cell-fate outcomes (6). Other reports have shown that caspase 3 regulates differentiation indirectly by targeting and inactivating proteins that maintain stem-cell self-renewal/pluripotency (8, 9). Although these later interactions contribute to the caspase effect, the targeting of these proteins does not explain the widespread alterations in gene expression and genomic reprogramming that typify differentiation.

One hallmark of caspase-mediated apoptosis that may also propel cell differentiation is chromatin alteration and DNA damage/fragmentation. A select handful of nucleases have been implicated in apoptotic DNA fragmentation, and caspase-activated DNase (CAD) is the most characterized (10). CAD is held in check through association with its inhibitor, inhibitor of caspase-activated DNase (ICAD), which is cleaved by caspase 3 to release CAD (10, 11). After activation, unobstructed CAD configures into a scissor-like dimer, cleaving DNA with minimal sequence specificity (10, 12). CAD is also ubiquitously expressed, suggesting a highly conserved function for this nuclease.

Although DNA damage is considered to be detrimental to cell integrity, DNA strand breaks have been shown to coordinate physiologic changes in gene expression independent of cell death. For example, V(D)J recombination is a mechanism in which DNA strand breaks precede a recombination event to generate variable expression of antigen receptor genes (13). In addition, a recent study has suggested that glucocorticoid-mediated gene expression is dependent on the formation of a strand break in target promoter elements; the DNA damage acts to facilitate nucleosome reorganization toward an active conformation, allowing gene expression to proceed (14). Interestingly, the appearance of transient DNA strand breaks during in vitro skeletal muscle differentiation and in vivo regeneration have been documented (15–17). Although the role of DNA damage was not investigated, these studies suggested an intriguing hypothesis that the differentiation process may be dependent on controlled DNA damage events.

Here, we investigated the role of caspase 3/CAD-mediated DNA strand breaks as a prerequisite for inducing cell differentiation. We show that the caspase activation of CAD participates in the formation of transient genome-wide DNA damage/strand breaks during early stages of the skeletal muscle differentiation. Reduction in CAD leads to the absence of strand-break formation with a near complete blockade of differentiation. In turn, we show that caspase 3/CAD activity induces strand breaks in the promoter of a critical cell-cycle regulatory factor p21, and these strand breaks are associated with the induction of p21 gene expression.

Results and Discussion

Transient DNA Strand Breaks Are Detectable During Myoblast Differentiation. We initially monitored DNA strand-break formation in individual myoblasts using single-cell gel electrophoresis (COMET assay). Myoblasts displayed evidence of DNA strand breaks between 12 and 48 h after low-serum induction of differentiation, and these strand breaks were qualitatively different from the DNA fragmentation associated with apoptosis (Fig. S1). This biochemical assay showed accumulation of DNA damage during myoblast differentiation; however, this assay was limited in the

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ability to discriminate DNA damage associated with healthy or early apoptotic cell types.

Therefore, to confirm the cellular status associated with the DNA strand-break formation, we used in situ nick translation (ISNT) coupled with fluorescence microscopy. This technique uses the enzymatic ability of DNA polymerase I to recognize and repair single-stranded (ss) DNA breaks in cells and to incorporate labeled nucleotides (15, 18, 19). Applying this technique to proliferating C2C12 myoblasts showed no incorporation of the labeled nucleotide, yet induction of apoptosis with Actinomycin D or after fixation treatment with DNase I showed robust nuclear incorporation (Fig. S2). Subsequently, ISNT was used to monitor the appearance of strand breaks during the progression of C2C12 and the primary myoblast-differentiation program. This protocol was coupled with DAPI nuclear counterstain to assess nuclear integrity. Within 12 h of low-serum induction of differentiation, a significant portion of the healthy nonapoptotic myoblast population, both in C2C12s and primary cells, displayed robust label incorporation (~70%) Fig. 1 *A* and *C*). Strand-break formation was transient, and C2C12 cells reached an apex early in the differentiation program (85% of muscle cells at 24 h) followed by an equally rapid disappearance of strand-break formation by 48 h (Fig. 1 *A* and *C*). Primary muscle cells displayed a more rapid disappearance of strand breaks by 24 h. Collectively, these results show that the viable muscle-cells population is subject to extensive DNA damage/strand-break formation during differentiation.

The transient nature of the DNA nick/strand breaks suggested that an active DNA repair process was engaged. Ataxia-telangiectasia mutated (ATM) is a member of a serine/threonine kinase family that consists of Ataxia-telangiestasia and Rad3 related (ATR) and DNA-protein kinase (PK), all of which respond to DNA strand breaks (20). ATM/ATR/DNA-PK phosphorylates and activates DNA repair proteins such as histone 2A family member X (H2AX), which act to stabilize the multiprotein complexes that facilitate the repair of DNA strand breaks (21). To examine the activation of the DNA repair process, the phosphorylation of the histone H2A variant H2AX was examined. Phospho-H2AX foci were observed in the vast majority of the differentiating muscle cells, suggesting an active and coordinated repair of strand breaks during myoblast differentiation (Fig. 1*B*). Importantly, we noted an absence of ISNT-detectable DNA strand breaks and phospho-H2AX foci in HeLa cells when transferred to low-serum conditions (Fig. S3); this suggests that the low-serum conditions used to differentiate myoblasts did not induce the observed DNA strand breaks and subsequent repair.

Caspase 3 Induces DNA Strand Breaks Through the Activation of CAD. Caspase 3 activity has been shown to be a cell-autonomous requirement for skeletal myoblast differentiation with an activation profile that parallels the formation of the observed DNA strand breaks (6). When differentiating C2C12 myoblasts were treated with caspase 3 peptide inhibitors, the ensuing blockade in myoblast differentiation was accompanied by an almost complete absence of DNA strand-break formation as detected by ISNT (Fig. 2*A* and Fig. S4). In addition, there was a notable reduction in the DNA damage-repair response after caspase 3 inhibition, which was measured by the reduction in number of phosphorylated H2AX positive nuclei accompanied with a visual decrease in the intensity of the staining in the remaining positive nuclei (Fig. 2*B*). We speculate that the ability of the cell to monitor and detect internal DNA strand breaks and elements of the repair process, as measured by phosphorylation of H2AX, seems more qualitative that the ISNT technique in fixed cells. As such, labeling of H2AX is able to detect DNA strand breaks at a lower threshold. These results establish that caspase 3 activity initiated the formation and eventual repair of DNA strand breaks during myoblast differentiation.

We next examined whether or not the caspase 3-mediated DNA damage response was associated with activation of CAD. CAD exists in complex with its inhibitor protein, which is referred to as ICAD. Activated caspase 3 cleaves ICAD at two aspartic acid residues (D117 and D224), releasing CAD from inhibition and allowing the DNase to target and fragment DNA (10). Partial ICAD cleavage at D117 was observed during early stages of myoblast differentiation, which corresponds to the period of DNA strand-break formation (Fig. 2*C*). The cleavage of ICAD at D117 has been shown to be necessary and sufficient for the release of CAD activity during apoptosis (11). We observed that inhibition of caspase 3 activity attenuated the cleavage of ICAD during myoblast differentiation, confirming that the ICAD partial cleavage stemmed from caspase 3 activity (Fig. 2*D*). No change in CAD expression was observed through the time course examined

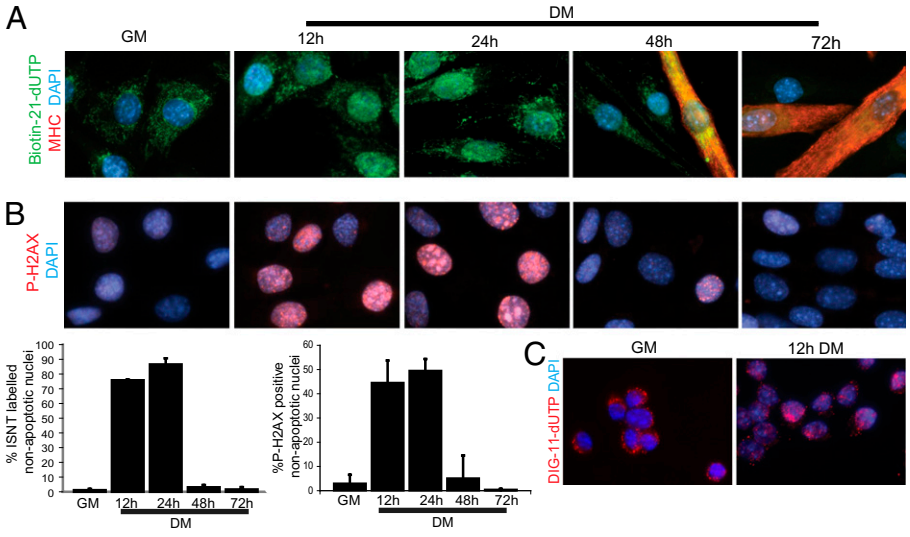

Fig. 1. Transient DNA strand breaks are detected early during C2C12 myoblast differentiation. (*A*) ISNT labels methanol-fixed C2C12 nuclei at 12 and 24 h after being switched from growth media (GM) to low-serum differentiation media (DM). Cells were stained for incorporated biotin-21-dUTP (green), myosin heavy chain (MHC; red), and DAPI (blue). (Error bars are SD.) (*B*) Phosphorylated H2AX foci are detected in differentiating C2C12 myoblasts at 12 and 24 h after low-serum–induced differentiation. Cells were fixed with 95% ethanol and 5% acetic acid and stained for P-H2AX (red) and DAPI (blue). (Error bars are SD.) (*C*) ISNT labels fixed primary myoblast nuclei at 12 h when switched to DM. Cells were stained for incorporated DIG-11-dUTP (red) and DAPI (blue).

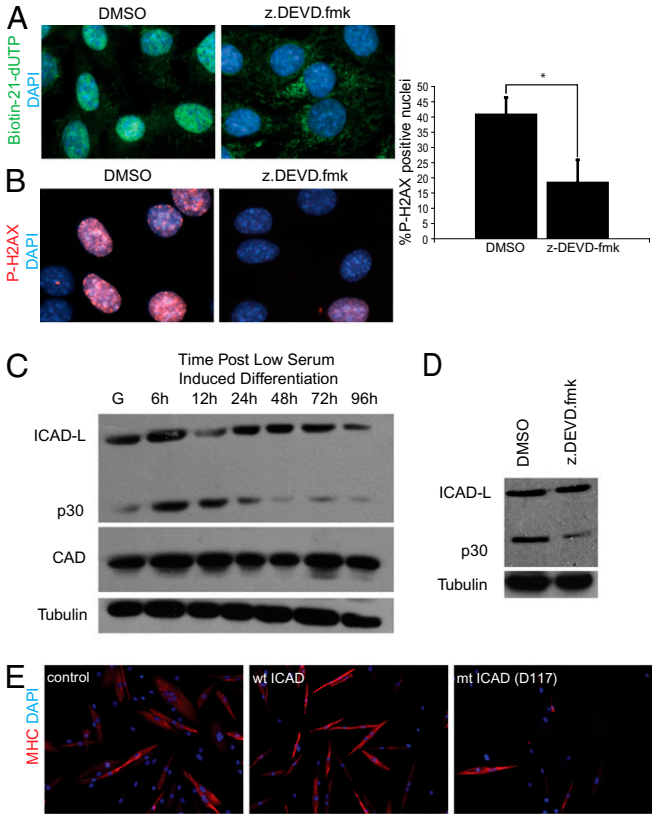

Fig. 2. DNA strand breaks are dependent on caspase 3 activity. (*A*) The caspase 3 inhibitor, z.DEVD.fmk, blocks the formation of DNA strand breaks detectable by ISNT. C2C12 myoblasts were induced to differentiate under low-serum conditions treated with either 10 μM z.DEVD.fmk or DMSO, and cells were fixed and stained as in Fig. 1*A*. (*B*) Formation of P-H2AX foci at 24 h of low-serum differentiation are diminished when myoblasts are treated with z.DEVD.fmk. Cells were treated as in *A* and fixed and stained as in Fig. 1*B*. (Error bars are SD; $P < 0.001$.) (*C*) Partial cleavage of ICAD corresponds to the transient formation of DNA strand breaks in differentiating C2C12 myoblasts. The ratio of p30 cleavage fragment to full-length ICAD increase by 4.6-fold at 12 h compared with growth. Protein lysates were collected from differentiating C2C12 myoblasts, and Western blot analysis for ICAD and CAD was performed. Tubulin was used as a loading control. (*D*) Caspase 3 inhibition impairs the partial cleavage of ICAD observed during differentiation. C2C12 myoblasts were differentiated for 12 h with 10 μM z.DEVD.fmk or DMSO. Protein lysates were collected, and Western blot analysis for ICAD was performed. (*E*) Expression of ICAD mutated at D117 impairs myoblast differentiation. Myoblasts were transfected with empty pMEV-2HA (control), wild-type–hICAD, or mutant D117E-hICAD. Stable expression was selected with G418, and then, myoblasts were induced to differentiate under low-serum conditions. Differentiation was assessed by the up-regulation of myosin heavy chain (red).

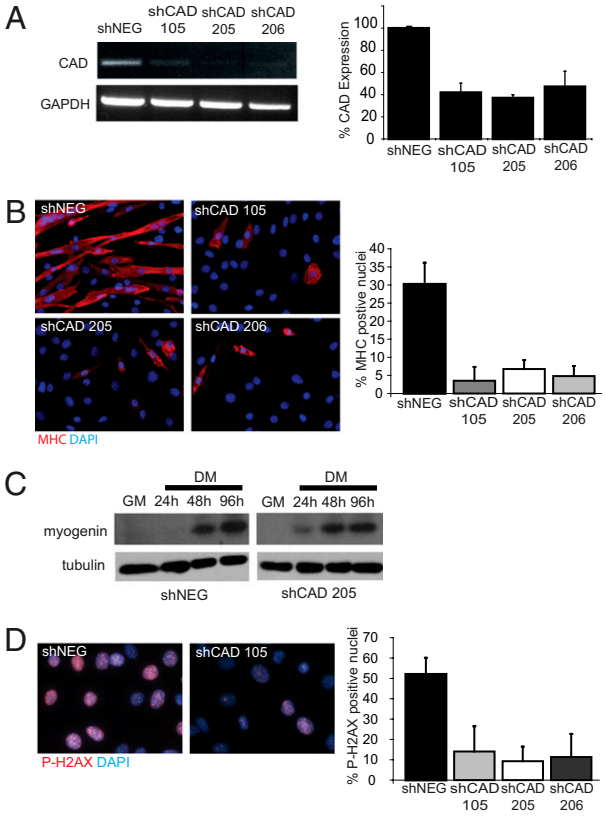

Fig. 3. Stable knockdown of CAD in C2C12 myoblasts affects differentiation. (*A*) RT-PCR for CAD showing knockdown in stable C2C12 clones with shCAD compared with a C2C12 clone with a negative-control shRNA vector. RT-PCR for GAPDH was used as a loading control. (Error bars are SEM.) (*B*) Up-regulation of myosin heavy chain is impaired in the stable cell lines compared with negative control. Cells were induced to differentiate for 72 h, fixed, and stained for MHC (red). MHC-positive nuclei were counted from three independent experiments. (Error bars are SD.) (*C*) Myogenin expression during differentiation is not affected in the stable cell lines. Protein lysates were collected from differentiating stable cell lines, and Western blot analysis for myogenin expression was performed. Tubulin was used as a loading control. (*D*) Formation of phosphorylated H2AX foci at 24 h differentiation is diminished in shCAD stables compared with negative control. Cells were induced to differentiate for 24 h, fixed, and stained for P-H2AX.

(Fig. 2*C*). In addition to ICAD cleavage, we also measured global nuclease activity during myoblast differentiation. Interestingly, lysates derived from differentiating myoblasts displayed temporally sensitive nuclease activity that was consistent with the formation of the caspase-sensitive DNA nicks/breaks (Fig. S5). The partial cleavage of ICAD at D117 lead us to assess the functional significance that this event played during myoblast differentiation by generating pooled stable myoblasts expressing the wild-type or mutant D117E human ICAD. Overexpression of the mutant ICAD adversely impacted differentiation as shown by impaired myosin heavy-chain expression and myotube formation (Fig. 2*E*).

To directly assess the role of CAD in the caspase-mediated differentiation signal, we generated stable myoblast cell lines expressing a short hairpin RNA directed to CAD. The stable myoblast cell lines displayed between 50% and 60% reduction in CAD expression compared with the negative control shRNA cell line (Fig. 3*A*). These cells were affected by a profound inhibition in the differentiation program as measured by the failure to generate myosin heavy-chain positive myotubes (Fig. 3*B*). Interestingly, up-regulation of the transcription factor myogenin was unaffected in the CAD shRNA clones, suggesting that CAD regulation of muscle gene expression seems to be specific for a subset of differentiation specific loci (Fig. 3*C*). This observation is consistent with previous reports, which show that inhibition of caspase 3 activity limited myoblast differentiation without negatively impacting myogenin expression (6). The impaired differentiation program in the CAD knockdown cell lines was also preceded by a substantial reduction in the formation of strand breaks (Fig. S6) and a corresponding absence of detectable DNA repair events as measured by phosphorylated H2AX foci (Fig. 3*D*). Collectively, these results strongly implicate CAD nuclease activity in promoting skeletal myogenesis through direct DNA damage/strand-break formation.

Up-Regulation of the Cell-Cycle Inhibitor p21 Requires CAD. The profound blockade in differentiation coupled with the failure to reduce myogenin expression in the CAD knockdown myoblasts

strongly suggests that CAD directs differentiation by activating a general mechanism rather than engaging a tissue-specific or lineage-restricted gene-expression program. One family of proteins that acts as a critical regulator of cell differentiation in this regard is the cyclin/cyclin-dependent kinase (CDK) inhibitors. For example, the increased expression of the CDK inhibitor p21 (p21CIP/WAF1) is indispensable for differentiation in numerous cell types including skeletal myoblasts, yet it is not directly dependent on muscle transcription-factor activity (22, 23). Compounds that induce apoptosis or cell-cycle arrest (UV or 5-azacytidine) have been shown to induce the expression of p21 while inflicting DNA damage within the p21 promoter (24–26). We noted that the normal up-regulation of p21 gene expression was impaired in the CAD shRNA clones, which prompted the examination of a direct role for CAD in regulating p21 expression (Fig. 4 *A* and *B*).

Activated CAD as well as the CAD–ICAD complex has been shown to form stable complexes with substrate DNA, suggesting that ICAD may be cleaved by caspase 3 in this DNA-associated complex to activate CAD in a specific manner (12). This stable association allowed for ChIP with an antibody specific for CAD to precipitate associated genomic regions at 12 and 24 h postinduction of differentiation in C2C12 myoblasts. CAD showed strong enrichment (~2.5-fold) at the p21 proximal promoter at 12 and 24 h differentiation (Fig. 4*C*). Examination of the myogenin promoter showed no enrichment for CAD at these time points. To localize the formation of strand breaks in the p21 promoter, we used a modified ligation-mediated PCR (LM-PCR) protocol; LM-PCR has been used to map DNA strand breaks that occur during apoptosis as well as site-specific strand breaks that result from glucocoticoid stimulation of Michigan Cancer Foundation (MCF)-7 cells (14, 27). Briefly, the DNA linker is ligated to a DNA strand break. Then, directed PCR, with primers that overlap the genomic region of interest, and the DNA linker amplify the target region of interest. Subsequently, the PCR product is radiolabeled and electrophoresised on a sequencing gel to visualize the location of DNA strand breaks. Within 12 h of low-serum induction of differentiation, LM-PCR revealed a number of strand breaks in the p21 promoter occurring within −200 bp of the transcriptional start site (Fig. 4 *D* and *F*). The extent of the strand breaks observed during differentiation were markedly reduced compared with those observed under apoptotic conditions, which further suggests that strand-break formation during differentiation is a well-controlled event. Interestingly, reduction in CAD or inhibition of caspase 3 activity during myoblast differentiation led to attenuated formation of strand breaks, suggesting that caspase/CAD activity was the primary determinant in strand-break formation (Fig. 4 *D* and *F*). Although we observed consistent formation of DNA strand breaks within the proximal region of the p21 promoter, we noted the positioning of the prominent break differs by ~50 bp between the primary versus C2C12 differentiating myoblasts. This variable positioning in the strand break is likely derived from an epigenetic influence related to the different genetic origin of the primary versus C2C12 myoblasts. Indeed, during apoptosis, CAD itself is known to retain only limited (if any) DNA sequence specificity; however, the nuclease seems to rely on nuclear architecture to position strand breaks. We further examined the myogenin promoter and found no evidence of strand breaks within −200 bp of the transcriptional start site during the induction of differentiation (Fig. 4 *E* and *G*).

Collectively, our observations suggest that CAD drives myoblast differentiation by targeting DNA strand breaks to specific genomic loci. However, studies have reported that genotoxic stress, which triggers random DNA damage, can induce premature differentiation (28, 29). To test this supposition, we treated myoblasts with neocarzinostatin, a compound that induces indiscriminate DNA double strand breaks. Interestingly, we noted that neocarzinostatin treatment leads to only sporadic myoblast differentiation, confirming that random DNA damage per se is not a developmental trigger for inducing the differentiation program (Fig. S7).

These observations suggest that modification of the p21 promoter by transient strand-break formation acts as an inductive event to establish p21 gene expression. This supposition is supported by the observation that transient formation of caspase 3/CAD-dependent breaks within the p21 promoter element are concurrent to the up-regulation of p21 in muscle differentiation and our observation of the impaired expression of p21 in CAD shRNA myoblasts. Although the mechanism by which the strand break leads to gene activation has yet to be elucidated, the caspase/CAD strand break may act to initiate histone modifications and/or changes in histone composition that ensure a more permissive gene-expression environment. Alternatively, the strand breaks and the subsequent repair process may act to remove DNA marks that are repressive. In this regard, we observed that, in normal proliferating C2C12 myoblasts, MspI restriction enzyme digest of the p21 promoter displayed an identical corresponding strand break between −89 and −90 bp from the transcription start site as that observed in early differentiating C2C12 myoblasts (Fig. S8*A*). However, we have not observed any alterations in the methylation status of the p21 promoter during myoblast differentiation (Fig. S8*B*). As such, we anticipate that the strand breaks in the p21 gene may act as points of assembly for the transcriptional machinery in a manner similar to the topoisomerase-II induced strand-break transcription that has been reported for glucocorticoid-sensitive gene expression (14).

The results presented here establish the induction of caspase 3/CAD-dependent DNA strand breaks as a primary end point in the caspase signal that propels differentiation. This signaling end point coupled with caspase-directed activation of select kinase substrates, such as MST1, may act in concert to precipitate the alterations in gene expression that promote myoblast differentiation. However, these caspase-dependent activities do not preclude a role for additional caspase signaling events in regulating cell-fate alterations. For example, the human tudor staphylococcal nuclease, HsTSN (p100), has been identified as a phylogenetically conserved substrate of caspase 3 (30). Cleavage of HsTSN by caspase 3 impairs mRNA splicing during apoptosis, an event that may also be a prerequisite for cell differentiation (30). Alternatively, HsTSN may function in a manner analogous to CAD as a caspase-responsive nuclease.

Despite the evidence that CAD seems to target discrete loci, the molecular mechanism that dictates CAD genome specificity remains unknown. CAD targeting preference may originate with simultaneous chromatin/epigenetic modifications that either promote and/or limit CAD accessibility to DNA. Alternatively, CAD may interact with a protein or proteins that direct the nuclease to a discrete genomic location. Our observations implicate caspase/CAD induction of p21 gene expression, yet the pattern of strand-break formation and repair (ISNT-labeled foci and phospho-H2AX) during myoblast differentiation is consistent with a genome-wide modification rather than a targeting of a single loci. Previous studies have linked CAD targeting to regions of DNA that have equitable distribution, such as accessible intranucleosomal linker regions and/or matrix attachment regions (MARs) (10, 27). Depending on the context of these targeted regions, one would predict that the CAD-directed cleavage events may be repressive for some genes/genomic loci but inductive for others. In this manner, caspase/CAD activity would provide an epigenetic mechanism to rapidly modify global gene-expression patterns, an event entirely consistent with the genome reorganization that occurs during differentiation (31). As such, our results conclude that caspase/CAD-regulated DNA strand breaks may be a broadly conserved mechanism for managing the genome alterations that promote cell differentiation.

Materials and Methods

Cell Culture. C2C12 myoblasts were cultured and induced to differentiate as described in Fernando et al. (6) For caspase 3 inhibition, myoblasts were pretreated with 10 μM N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD-fmk) for 2–3 h before the induction of low-serum

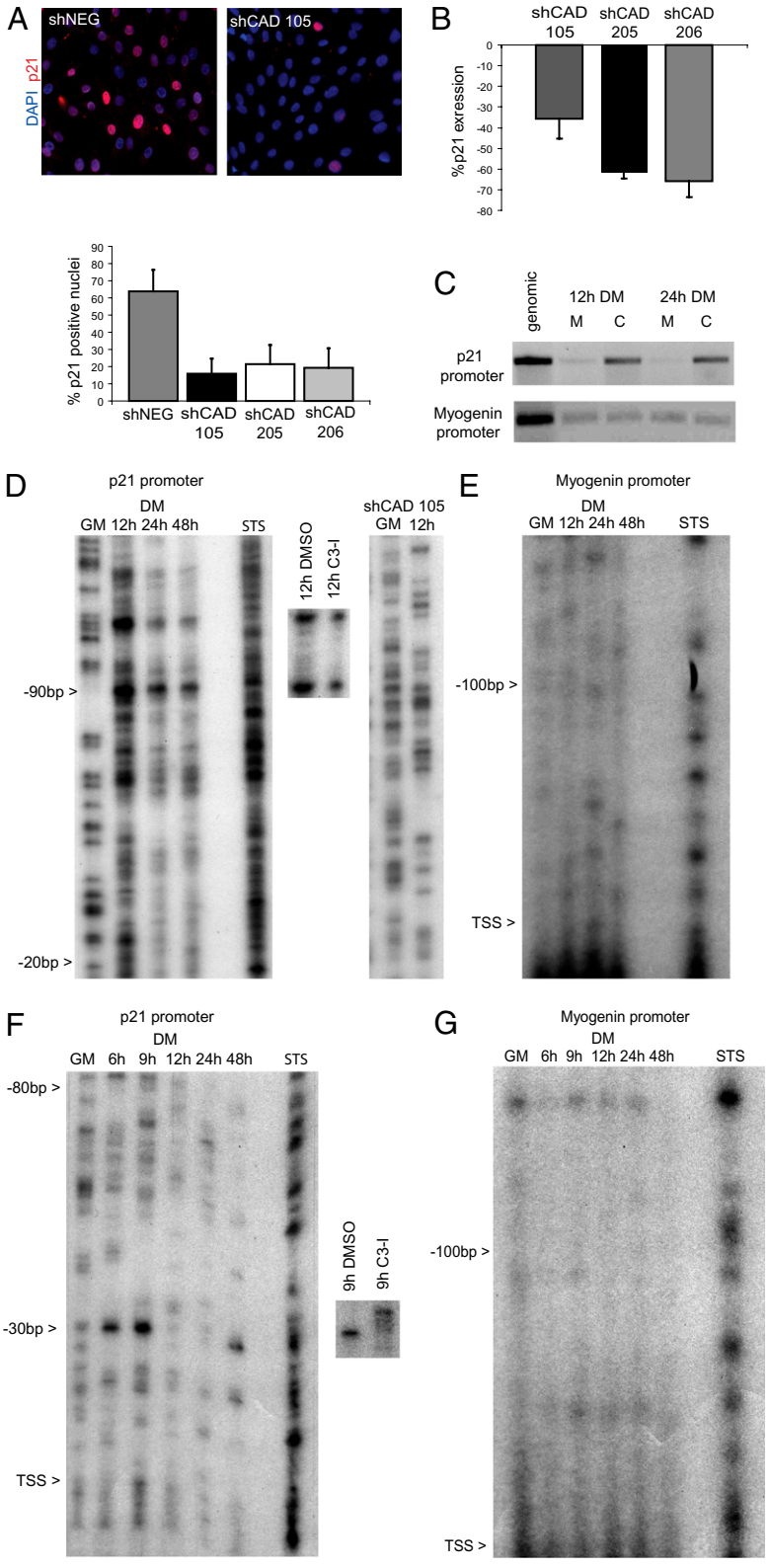

Fig. 4. Caspase 3/CAD inflicts DNA strand breaks within the promoter of the cell-cycle inhibitor p21. (*A*) Up-regulation of the cell-cycle inhibitor p21 is reduced in the stable shCAD cell lines compared with negative control. Cells were induced to differentiate for 24 h, fixed, and stained for p21 (red). Nuclei were counterstained with DAPI (blue). p21 positive nuclei were counted from three independent experiments. (Error bars are SD.) (*B*) p21 transcripts levels are reduced in the shCAD cell lines compared with negative control. RNA was isolated 24 h after the induction of differentiation and subject to semiquantitative RT-PCR. Multiplex PCR to p21 and GAPDH cDNA was run in triplicate for an $n = 3$ experiment. Percent reduction in p21 transcript in the CAD knock down (KD) myoblasts is compared with the negative control line. (*C*) CAD associates with the p21 promoter during C2C12 myoblast differentiation. ChIP using rabbit IgG (M) or an antibody against CAD (C) shows enrichment at the p21 promoter at 12 (2.5-fold) and 24 h (2.6-fold) after the induction of differentiation; no enrichment was observed of the myogenin promoter. (*D* and *F*) LM-PCR delineates the formation of DNA strand breaks within the p21 promoter of differentiating C2C12 and primary muscle cells. Genomic DNA was isolated from proliferating, differentiating, and apoptotic (staurosporine [STS]) muscle cells and subjected to LM-PCR. Primers specific to the p21 promoter allowed analysis of ~200 bp before the transcriptional start site (TSS; $n = 3$). (*D Inset* and *F Inset*) Chemical inhibition of caspase 3 reduces the formation of strand breaks within the p21 promoter. Differentiating C2C12 and primary cells were treated with either 10 μM z.DEVD.fmk or DMSO; genomic DNA was extracted and subjected to LM-PCR ($n = 3$). Reduction in CAD expression impairs the formation of strand breaks within the p21 promoter in genomic DNA isolated from shCAD105 cells under low-serum conditions (*D Inset*; $n = 2$). (*E* and *G*) LM-PCR shows the absence of robust DNA strand breaks in the myogenin promoter during differentiation of C2C12 and primary muscle cells. Primers specific to the myogenin promoter allowed analysis of ~200 bp before the TSS ($n = 3$).

differentiation when fresh inhibitor was added; inhibitor was refreshed every 24 h. Primary myoblasts were isolated from 4-week-old B6C3F1 mice, cultured, and induced to differentiate as described in Fernando et al. (6)

ISNT. C2C12 myoblasts were cultured on glass coverslips and fixed with 90% methanol and 10% PBS after indicated treatment. C2C12 myoblast apoptosis was induced by treatment with 1 mM Actinomycin D for 12 h at 37 °C or 1 μg/mL anti-Fas monoclonal antibody for 4 h before fixation. For DNase I New England Biolabs [NEB] treatment, fixed cells were incubated with 2U DNase I for 10 min at 37 °C. Nick translation reaction [50 mM Tris HCl (pH 7.9), 50 μg/mL BSA, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10 μM dCTP, 10 μM dATP, 10 μM dGTP, 1 μM dTTP, and 1 μM dUTP-21-Biotin (Clontech) or 1 μM dUTP-11-DIG (Roche)] was

run with 10 U/mL DNA Polymerase I (NEB) for 1 h at 37 °C. Subsequent detection of incorporated labeled dUTP was performed using appropriate primary antibody; where indicated, myosin heavy chain was stained to mark differentiation, and nuclei were counterstained with DAPI (Sigma).

ChIP. ChIP assays were run as described by Rampalli et al. (32). Primers to the p21 promoter were 5′-CCCGAAACCCAGGATTTTAT-3′ and 5′-TCCCCTCTGGGAATCTAAGC-3′. PCRs for the myogenin promoter were run using the following primer pairs: 5′-AGAGGGAAGGGGAATCACAT-3′ and 5′-ATAGAAGTGGGGCTCCTGGT-3′. PCR product amplification was quantified using AlphaEaseFC software (Alpha Innotech).

LM-PCR. LM-PCR was run as described by Carey and Smale (33) with some modifications. Briefly, genomic DNA was isolated using the Sigma Genlute Mammalian Genomic DNA Miniprep Kit. Primer extension using primer 1 was run with 500 ng of purified DNA using pfu polymerase (Biovision). Linker DNA was ligated overnight at 16 °C with T4 Ligase (Invitrogen). Amplification was run using primer 2 and Phusion DNA polymerase (NEB). ^{32}P-labeled primer 3 was used to label PCR products using Phusion DNA polymerase. PCR products were resolved on a 6% denaturing acrylamide sequencing gel. Primers to the p21 promoter were primer 1 (5′-ACTCCAATTCCCCTAGACTCTGA-3′), primer 2 (5′- CCAATTCCCCTAGACTCTGACAC-3′), and primer 3 (5′-GACTCT GACACCGCGGCGGCTCACACCTCT-3′). Primers to the myogenin promoter were primer 1 (5′-CTGAAGGTGGACAGGAAGGTAGTT - 3′), primer 2 (5′-TGTCTCATACAGCTCCATCAGGTCGG-3′), and primer 3 (5′-CAGCTCCATCAGGTCGGCAAAGGCTTGTTC -3′). Linker DNA 1 (5′-GCGGTGACCCGGGAGATCTGAATTC-3′) and linker DNA 2 (5′-GAATTCAGATC-3′) were annealed by heating to 98 °C for 5 min and then cooling gradually to room temperature.

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