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Dysregulation of the intrinsic apoptotic pathway mediates megakaryocytic hyperplasia in myeloproliferative neoplasms

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ABSTRACT

Aims Megakaryocyte expansion in myeloproliferative neoplasms (MPNs) is due to uncontrolled proliferation accompanied by dysregulation of proapoptotic and antiapoptotic mechanisms. Here we have investigated the intrinsic and extrinsic apoptotic pathways of megakaryocytes in human MPNs to further define the mechanisms involved.

Methods The megakaryocytic expression of proapoptotic caspase-8, caspase-9, Diablo, p53 and antiapoptotic survivin proteins was investigated in bone marrow specimens of the MPNs (n=145) and controls (n=15) using immunohistochemistry. The megakaryocyte percentage positivity was assessed by light microscopy and correlated with the MPN entity, *JAK2*^{V617F}/*CALR* mutation status and platelet count.

Results The proportion of megakaryocytes in the MPNs expressing caspase-8, caspase-9, Diablo, survivin and p53 was significantly greater than controls. A greater proportion of myeloproliferative megakaryocytes expressed survivin relative to its reciprocal inhibitor, Diablo. Differences were seen between myelofibrosis, polycythaemia vera and essential thrombocythaemia for caspase-9 and p53. *CALR*-mutated cases had greater megakaryocyte p53 positivity compared to those with the *JAK2*^{V617F} mutation. Proapoptotic caspase-9 expression showed a positive correlation with platelet count, which was most marked in myelofibrosis and *CALR*-mutated cases.

Conclusions Disruptions targeting the intrinsic apoptotic cascade promote megakaryocyte hyperplasia and thrombocytosis in the MPNs. There is progressive dysfunction of apoptosis as evidenced by the marked reduction in proapoptotic caspase-9 and accumulation of p53 in myelofibrosis. The dysfunction of caspase-9, which is necessary for proplatelet formation, may be the mechanism for the excess thrombocytosis associated with *CALR* mutations. Survivin seems to be the key protein mediating the megakaryocyte survival signature in the MPNs and is a potential therapeutic target.

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are a group of clonal proliferative bone marrow diseases characterised by somatic mutations (eg, *JAK2*^{V617F}, *CALR* frameshift lesions)^{1–5} and varying hyperplasia of the myeloid lineages. Megakaryocyte hyperplasia with clustering and associated morphological atypia with pleiomorphism are key diagnostic histological

features.^{6–8} The pathobiological basis underlying these numerical and morphological megakaryocytic abnormalities is thought to result from multiple molecular disruptions promoting proliferation and enhancing survival.^{9–13} These megakaryocytes have impaired death mechanisms conferred by overexpression of antiapoptotic Bcl-XL and reductions in pro-death BNIP-3.^{9 11–13} These changes are universal in the MPNs but there are differences between entities. Megakaryocytes in essential thrombocythaemia (ET) have been shown to have a more proliferative profile, whereas in myelofibrosis (MF) they exhibit greater proapoptotic impairments.^{9 13} These changes occur irrespective of the *JAK2*^{V617F} or calreticulin (*CALR*) driver mutations, although those with a *CALR* lesion have greater proapoptotic dysfunction.¹³ The mechanisms driving this apoptotic dysregulation in megakaryocytes in the MPNs have not been explored.

Apoptosis is mediated via extrinsic and intrinsic apoptotic cascades (figure 1), with both pathways being capable of inducing programmed cell death following exposure to apoptotic insults and the accumulation of excess DNA damage.^{14–16} These pathways converge at the cleavage of procaspase-3 to produce active caspase-3, which is capable of committing the cell to apoptosis.^{14–16} Alterations in caspase biology, including caspase-8 (extrinsic) and caspase-9 (intrinsic), have been implicated in a number of malignancies in humans and animal models. In caspase-8-deficient mice, B-lymphocytes have impaired cytokinesis and chromosomal instability, and show a tendency towards lymphoma development.¹⁷ Similarly, caspase-9 gene polymorphisms and its downregulation are associated with solid tumours and their malignant progression.^{18–20} Both caspase-8 and caspase-9 are potentially important in regulating megakaryocyte turnover in the MPNs. Frameshift lesions targeting *CALR* may disrupt megakaryocyte apoptosis through its inability to facilitate caspase-8 activation and antiapoptotic protein cleavage.²¹ While the precise pathogenesis surrounding *CALR* lesions and megakaryocyte apoptosis is unknown, its mutated product is exclusively expressed in myeloproliferative megakaryocytes.^{22 23} In megakaryocytes, caspase-9 activity appears necessary for proplatelet formation.²⁴ Some reports suggest that caspase-9 may be redundant, with *CASP9* deletions in *ex vivo*-cultured mouse megakaryocytes impairing procaspase-3 activation, and



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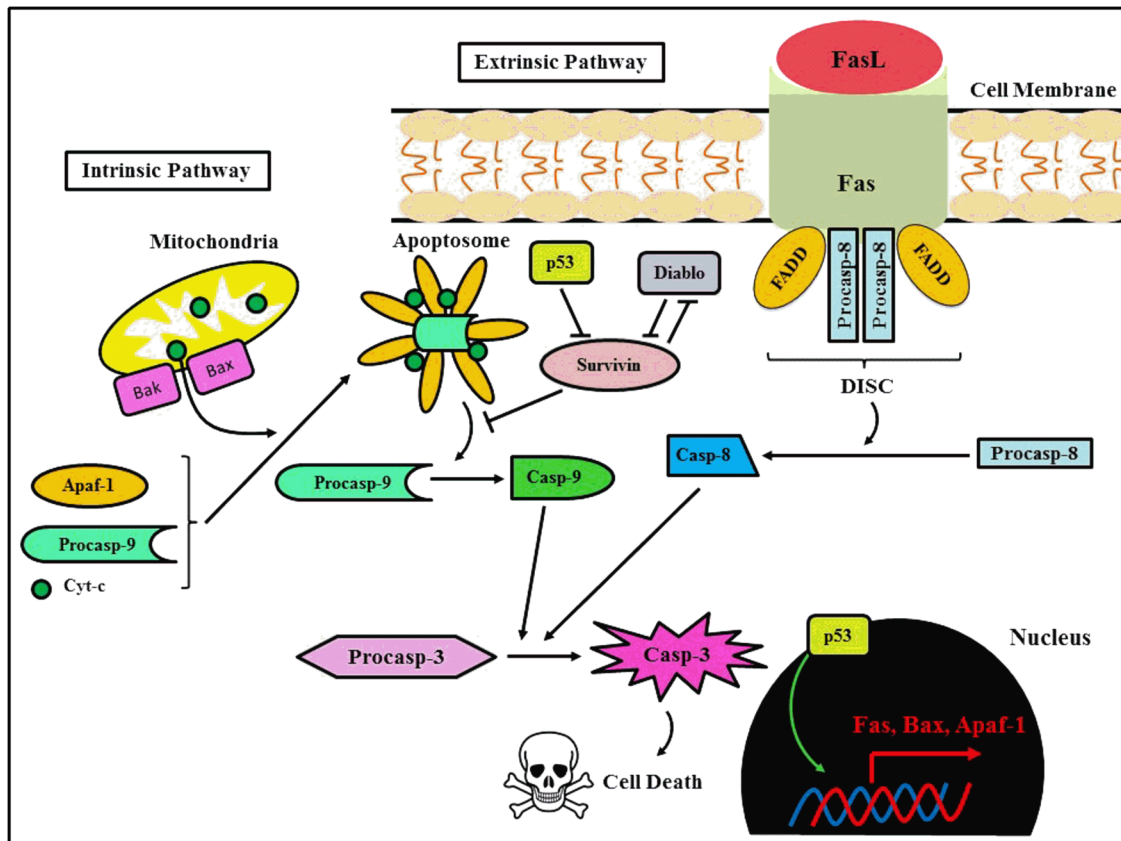


Figure 1 Diagrammatic representation of the extrinsic and intrinsic apoptotic pathways. The extrinsic (receptor-mediated) apoptotic pathway commences following FasL binding the Fas death receptor. The union forms a death complex that is capable of recruiting FADD and procaspase-8 to form the DISC. The DISC recruits procaspase-8 proteins that undergo reciprocal cross-proteolysis and proximity-induced dimerisation to form an active caspase-8 that targets procaspase-3. The intrinsic (mitochondrial-mediated) apoptotic pathway relies on the insertion of homo-oligomerised pro-death Bcl-2-family proteins (Bak and Bax) into the outer mitochondrial membrane. The release of cytochrome-c subsequently ensues and acts to recruit procaspase-9 and Apaf-1 to form the heptameric apoptosome. The apoptosome recruits procaspase-9 molecules, which reciprocally cleave one another to form an active caspase-9 that targets procaspase-3. Both caspase-8 and caspase-9 then cleave procaspase-3 to form the executioner caspase-3, which brings about coordinated cell death. Survivin aims to inhibit caspase-9 activation while simultaneously competing with the inhibitory action of cytosolic p53 and Diablo. Nuclear p53 also activates the transcription of pro-death molecules required in both apoptotic cascades. Apaf-1, apoptotic protease activating factor-1; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; Casp-3, caspase-3; Casp-8, caspase-8; Casp-9, caspase-9; Cyt-c, cytochrome-c; DISC, death-inducing signalling complex; FADD, Fas-associated protein with death domain; Fas, Fas death receptor; FasL, Fas receptor ligand; Procasp-3, procaspase-3; Procasp-8, procaspase-8; Procasp-9, procaspase-9.

encouraging both megakaryopoiesis and proplatelet formation.²⁵ This does not however discount the presence of other caspases to conduct cytoskeletal proplatelet fragmentation in the absence of caspase-9. Moreover, caspase-9 loss may enhance the tumourigenic potential of megakaryocytes as their proliferative capacity increases.²⁵

Apoptotic cell death via the intrinsic pathway is regulated by inhibitors of apoptosis proteins (IAP) that specifically constrain the pro-death actions of caspase-9. One of these IAP, survivin, restricts the IAP-inhibitor Diablo protein and prevents it from activating caspase-9.^{26–29} This impedes the intrinsic apoptotic pathway and confers a cytoprotective effect. The relevance of Diablo has been shown in human tumours: downregulation has been associated with progressive disease and poor survival in both solid and haematological malignancies.^{30–33} Survivin over-expression is well documented in leukaemias^{34–37} and lymphomas^{38–41} where it increases the survival capacity of affected tumour cells. On the other hand, survivin loss causes mitotic catastrophe characterised by cell death or polyploidisation.^{37, 42} Mouse megakaryocytes failing to express survivin have limited proliferation but increased ploidy, with loss of the IAP

preferentially selecting for those megakaryocytes capable of escaping intrinsic apoptotic fates.⁴² Megakaryocytes are known to express survivin and during anaphase it restricts cytokinesis to enable polyploidisation.⁴³ The relationship between survivin, Diablo and caspase-9 is complex and has not been assessed in megakaryocyte survival in the MPNs.

p53, the ‘master’ regulator of cell cycle, is also involved in the apoptotic machinery. Its regulatory role involves arresting the cell cycle, initiating senescence and inducing DNA repair mechanisms.^{44, 45} Failing these, p53 activates the intrinsic apoptotic cascade by binding Bak/Bax to induce cytochrome-c release while simultaneously inhibiting antiapoptotic Bcl-2-related proteins^{46, 47} and survivin.⁴⁸ The role of p53 in megakaryocytes is disputed. *Ex vivo*-cultured megakaryocytes have been shown to express low levels of p53⁴⁹ and its absence in p53-null mice has no effect on megakaryopoiesis.⁵⁰ However, the *in vitro* knock-down of p53 has been reported to increase megakaryocyte proliferation and regulate both its ploidy and differentiation.^{51–54} This disparity between *in vitro* and *ex vivo* experimental settings ultimately suggests that *in vivo*, p53 loss is tolerated in the presence of other compensatory proapoptotic mechanisms. In the

MPNs, alterations in p53 have not been linked to megakaryocyte hyperplasia although mutations targeting *TP53* do occur during their leukaemic transformation.^{55–57}

Apoptotic signalling is crucial to megakaryocyte polyploidisation and platelet production. Limited studies have identified that apoptotic signalling processes are disrupted in megakaryocytes of the MPNs.^{9–13} We intended to further delineate the biological basis of the apoptotic disturbances affecting megakaryocytes in the MPNs by assessing several biomarkers implicated in the intrinsic and extrinsic apoptotic pathways. We demonstrate through immunohistochemical analyses of human MPNs that the enhanced survival of myeloproliferative megakaryocytes occurs through inhibition of intrinsic death effectors despite concurrent extrinsic apoptotic activation.

MATERIALS AND METHODS

Patient samples

Bone marrow trephine (BMT) specimens from patients with polycythaemia vera (PV), ET and MF (including both primary MF and post-PV and post-ET MF) (n=145) and normal bone marrow controls (n=15) were collected from patients through PathWest Laboratory Medicine (Western Australia, Australia) and Queen Mary Hospital (Hong Kong SAR, China) between 1999 and 2015. Of the 145 patients with MPNs, 133 were untreated at the time of bone marrow collection. The PathWest specimens were formalin-mercury fixed while those from Hong Kong were fixed in formalin. All BMT specimens were acid decalcified and paraffin embedded. Morphological review of all cases was undertaken in accordance with WHO criteria⁷ and classified according to MPN entity (table 1). PathWest BMT specimens were processed using a TMA Master tissue microarrayer (3DHitech, Australia) to create tissue microarrays (TMA) as outlined in Malherbe *et al.*¹³ Whole BMT sections from the Hong Kong cohort were used for immunohistochemical investigation. *JAK2*^{V617F} mutation testing was performed by allele-specific PCR analysis. *CALR* mutations were detected using methods outlined by Nangalia *et al.*⁵ *MPL* mutation testing was not performed and cases negative for *JAK2*^{V617F} and *CALR* mutations were classified as 'double negative' (DN). Platelet counts ($\times 10^9/L$) were recorded for each MPN case at the time of collection of the BMT specimen.

Immunohistochemical staining

Whole BMT specimens and TMA were sectioned at 4 μm onto charged glass slides (Hurst Scientific, Australia). Monoclonal antibodies were to formalin-mercury-resistant epitopes and were

validated on control tissue prior to their application on BMT sections. Antibodies used were to CD61 (clone 2f2, Leica Biosystems, Australia), caspase-8 (clone 90A992, Thermo Scientific Pierce Antibodies, Australia), caspase-9 (clone F-7, Santa Cruz Biotechnology, USA), p53 (clone DO-7, Leica Biosystems, Australia), survivin (clone 71G4B7, Cell Signaling Technologies, USA) and Diablo/Smac (clone D5S3R, Cell Signaling Technologies, USA). All immunohistochemical staining was performed on an automated Leica BOND RX immunostainer (Leica Biosystems) as outlined by Malherbe *et al.*¹³ Positive and negative megakaryocytes were counted by a minimum of two observers and the percent positive calculated for each apoptotic biomarker. Megakaryocyte-rich (≥ 50) areas within whole BMT specimens were selected for enumeration of each biomarker up to a maximum of 200 megakaryocytes. Observers were blinded to both the diagnostic entity and mutation status of cases. Tissue areas and/or megakaryocytes of interest were photographed using a Pixera Pro 600ES microscope camera (Pixera, USA).

Statistical analysis

Mean megakaryocyte percentage positivity and SD were calculated according to the MPN subtype and *JAK2*^{V617F}/*CALR* mutational status for each apoptotic biomarker. Significant differences between the MPNs and control megakaryocyte positivity were assessed using Mann–Whitney U tests. One-way Kruskal–Wallis analysis of variance (ANOVA) analyses with post hoc Dunn's tests were performed to evaluate megakaryocyte positivity variations in relation to subtype and *JAK2*^{V617F}/*CALR* mutation status. Platelet counts for the MPNs stratified according to subtype and *JAK2*^{V617F}/*CALR* mutation status were correlated with the mean megakaryocyte expression for all biomarkers using Spearman's correlation. Significant differences were set at $p < 0.05$. All statistical analyses were conducted using GraphPad Prism V.6 software (GraphPad Software, USA).

RESULTS

MPNs versus controls

Megakaryocytes were visually identified on morphology, antigen expression (red chromogen) and nuclear haematoxylin counterstain. All antibodies produced the expected expression profile; there was no non-specific background staining in any sections. CD61 was used to confirm the identity of megakaryocytes in BMT with marked atypia (eg, MF). Mean megakaryocyte positivity was significantly greater in the MPNs than in controls for all apoptotic biomarkers, that is, caspase-8, caspase-9, survivin, Diablo and p53 (figure 2). Caspase-8 showed significantly more (~16.8%) positive megakaryocytes in the MPNs than controls, $p = 0.0005$. A similar significant trend was observed for caspase-9, although this increase was smaller (~4.3%), $p = 0.023$. Of note was that megakaryocytes present within clusters, a key feature of the MPNs, showed strong positivity for both caspase-8 and caspase-9, whereas single intertrabecular or paratrabecular megakaryocytes were more commonly negative (figure 3A–C). There were significantly greater numbers of Diablo and survivin-positive megakaryocytes in the MPNs than controls, ~3.1-fold, $p < 0.0001$ and ~1.8-fold, $p < 0.0001$, respectively. When comparing the proportion of Diablo and survivin-positive megakaryocytes in the MPNs, the balance was in favour of survivin by ~19.0%. The cytoplasmic expression of Diablo was weak and heterogeneous among megakaryocyte clusters in the MPNs (figure 3D). Contrastingly, myeloproliferative megakaryocytes showed strong nuclear localisation of survivin

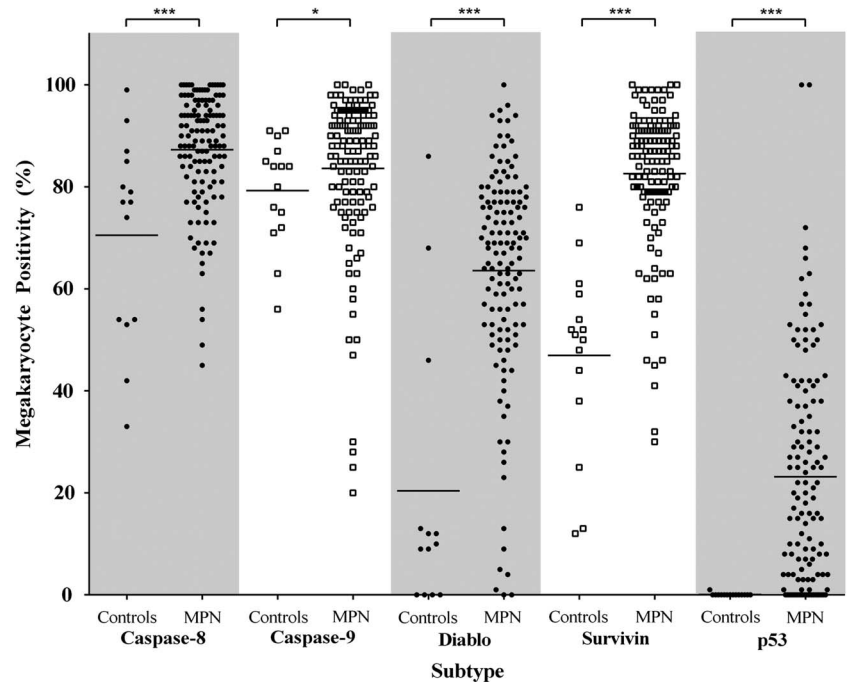
Table 1 Summary of the cohort studied

Subtype	<i>JAK2</i> ^{V617F} and <i>CALR</i> mutation status			Unknown	Total
	<i>JAK2</i> ^{V617F+}	<i>CALR</i> ^{Mut}	<i>JAK2</i> ^{V617F-} / <i>CALR</i> ^{WT}		
Controls	–	–	15	–	15
PV	20	–	–	–	20
ET	26	9	6	31	72
MF*	18	14	5	16	53
Total	64	23	26	47	160

JAK2^{V617F} and *CALR* lesions did not coexist (ie, *JAK2*^{V617F+} and *CALR*^{Mut} were mutually exclusive). All *CALR* mutations represent frameshift lesions in exon 9 of *CALR*.

*Includes both primary MF and ET/PV cases having undergone fibrotic transformation. *CALR*, calreticulin; ET, essential thrombocythaemia; *JAK2*, Janus activated kinase 2; MF, myelofibrosis; Mut, mutated; PV, polycythaemia vera; WT, wild type.

Figure 2 Percentage of positive megakaryocytes in myeloproliferative neoplasms (MPNs) and controls for caspase-8, caspase-9, Diablo, survivin and p53. Mean megakaryocyte positivity in MPN cases was significantly greater for all biomarkers in comparison to controls. Statistically significant difference, $p < 0.05$ (*), $p < 0.001$ (***)



(figure 3E, F). p53 was positive (nuclear) in myeloproliferative megakaryocytes (~23.1%) and virtually absent from controls, $p < 0.0001$ (figure 3G, H).

MPNs by disease entity and genotype

Since myeloproliferative megakaryocytes showed increased expression of apoptosis-associated antigens, we then proceeded to assess reactivity by morphological entity (ie, PV, ET and MF) and mutation status (ie, $JAK2^{V617F+}$, $CALR^{Mut}$ and DN: $JAK2^{V617F-}/CALR^{WT}$). Using Kruskal–Wallis ANOVA analyses, significant differences were seen between the MPN subtypes for caspase-9 and p53, $p = 0.0015$ and $p = 0.0079$, respectively (figure 4). Post hoc analyses showed caspase-9 expression to be significantly lower in MF megakaryocytes (~77.9%) than both PV (~90.3%, $p = 0.0032$) and ET (~85.6%, $p = 0.019$) with a similar, borderline insignificant trend for survivin, Kruskal–Wallis ANOVA $p = 0.073$. The number of p53-expressing megakaryocytes in MF was ~2.7-fold greater than in PV trephines, $p = 0.0071$. The differences in megakaryocyte positivity between the MPN entities for caspase-8 and Diablo were not significant, $p > 0.05$ (data not shown).

When apoptotic biomarkers were assessed by mutation status, no significant differences were seen, $p > 0.05$. The proportion of p53-expressing megakaryocytes was greater in $CALR^{Mut}$ than $JAK2^{V617F+}$ trephines, although this difference was borderline insignificant, Kruskal–Wallis ANOVA $p = 0.0660$, post hoc $p = 0.0667$ (figure 5). Caspase-8-positive and caspase-9-positive megakaryocytes in $JAK2^{V617F+}$ and $CALR^{Mut}$ cases were similar and marginally increased when compared with $JAK2^{V617F-}/CALR^{WT}$ (figure 5). Mean Diablo and survivin megakaryocyte positivity did not differ by genotype (data not shown).

Apoptotic markers and platelet count

Since proplatelet production requires megakaryocyte apoptotic signalling,^{24 25} platelet counts were correlated with megakaryocyte positivity for all apoptotic biomarkers in the MPNs. There was a significant, weak positive correlation between caspase-9 expression and platelet count for all MPNs, $r = 0.28$, $p = 0.0018$

(figure 6A). This correlation was strongest for cases of MF, $r = 0.34$, $p = 0.026$ (figure 6B) and most notably those MPNs with $CALR^{Mut}$, $r = 0.50$, $p = 0.030$ (figure 6C). When cases that had received prior therapy were excluded from this analysis, these correlations remained significant (data not shown). No significant correlations existed between any of the other apoptotic biomarkers and platelet count, $p > 0.05$ (data not shown).

DISCUSSION

Previous studies have indicated that there is dysfunction of apoptotic mediators in the MPNs, that is, increased antiapoptotic Bcl-XL and reduced pro-death BNIP-3.^{9 13} However, the precise apoptotic disruptions promoting abnormal megakaryocyte accumulation and thrombocytosis remain unclear. Here we provide insight into these mechanisms and demonstrate that the enhanced survival of megakaryocytes in the MPNs may be a result of dysregulation of the intrinsic apoptotic pathway. The key protein mediating this survival signature appears to be survivin, an inhibitor of the intrinsic apoptotic cascade.^{27–29} While survivin impedes megakaryocyte death, the reciprocal increase of Diablo, its inhibitor,²⁶ is insufficient and unlikely to nullify its antiapoptotic effect. Further, there is low-level upregulation of caspase-9, the main effector of the intrinsic apoptotic cascade (figure 1),¹⁶ and its expression correlated with platelet count. These data suggest that these aberrations facilitate megakaryocyte hyperplasia and thrombocytosis rather than directing apoptotic execution. The overexpression of pro-death caspase-8 (extrinsic) and p53 may be an attempt to counteract these changes. The progressive dysfunction of megakaryocyte apoptosis, which has previously shown to be most discernible in MF and $CALR$ -mutated MPNs,^{9 13} can now be further supported by marked reductions in pro-death caspase-9 and accumulation of p53.

Survival advantages gained through inhibition of the intrinsic apoptotic cascade is a key pathological mechanism promoting megakaryocyte hyperplasia in the MPNs. We have previously demonstrated that myeloproliferative megakaryocytes show disrupted expression of the Bcl-2 family of apoptotic biomarkers

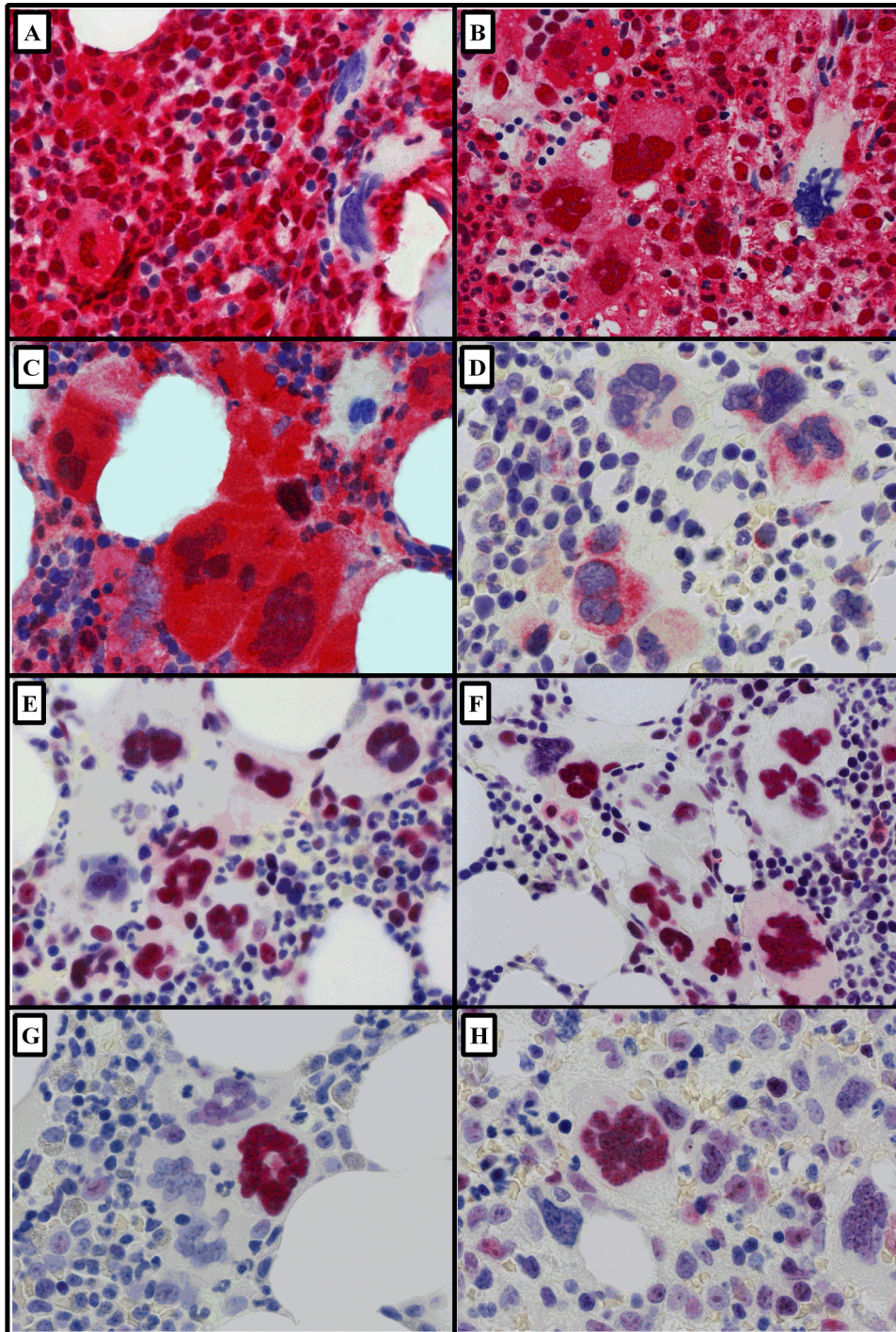


Figure 3 Representative images ($\times 600$) of immunohistochemically stained megakaryocytes in myeloproliferative neoplasms (bone marrow trephine sections; Fast Red chromogen and haematoxylin counterstain). (A) Caspase-8 in polycythaemia vera and (B) myelofibrosis (MF) and (C) caspase-9 in MF. (D) Diablo-positive megakaryocytes in essential thrombocythaemia (ET). Clusters of megakaryocytes showing strong positive nuclear expression for (E) survivin in ET and (F) in MF. In contrast, nuclear p53 was limited to isolated positive megakaryocytes among a negative majority (G) in ET and (H) in MF.

(ie, upregulated antiapoptotic Bcl-XL, diminished pro-death BNIP-3) that regulate intrinsic apoptosis.¹³ We further support this theory by showing that survivin is strongly overexpressed in myeloproliferative megakaryocytes. Survivin acts to limit procaspase-9 cleavage, thereby constraining caspase-9 activation in megakaryocytes and impeding their death (figure 1).^{27–29} Furthermore, survivin assists megakaryocyte polyploidisation and its increase in the MPNs may be an obligatory survival

prerequisite as the majority of these megakaryocytes are of high ploidy (up to 512N).^{43–58} Myeloproliferative megakaryocytes attempt to neutralise this survival advantage by expressing Diablo, which directly competes with the antideath effects conferred by excess survivin.²⁶ However, a greater proportion of megakaryocytes in both controls ($\sim 26.5\%$) and the MPNs ($\sim 19.0\%$) expressed survivin than those with upregulated Diablo content. Further, the weak expression intensity of

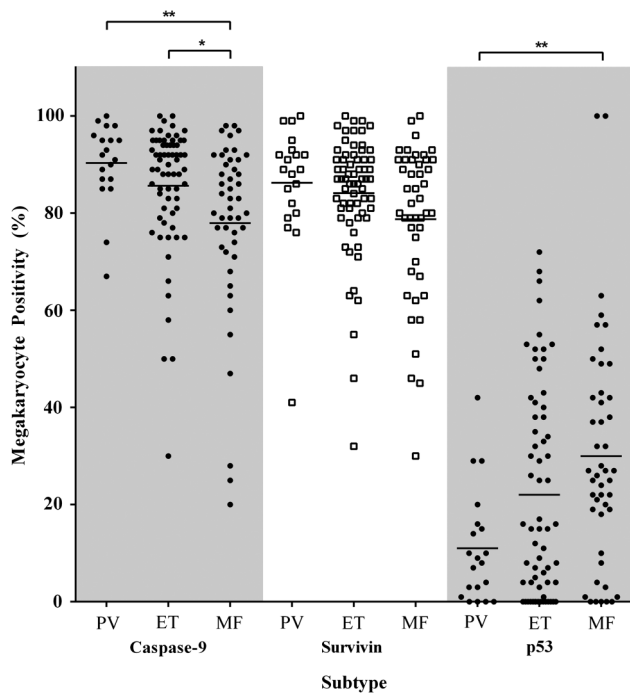


Figure 4 Percentage of megakaryocytes positive according to myeloproliferative neoplasm entity for caspase-9, survivin and p53. Mean megakaryocyte positivity for caspase-9 was significantly lower in myelofibrosis (MF) than polycythaemia vera (PV) and essential thrombocythaemia (ET), $p=0.0032$ and $p=0.019$, respectively. The per cent positive megakaryocytes in MF was lower for survivin ($p=0.073$) and higher for p53 than PV and ET, $p=0.0071$ and $p=0.18$, respectively. Statistically significant difference, $p<0.05$ (*), $p<0.01$ (**).

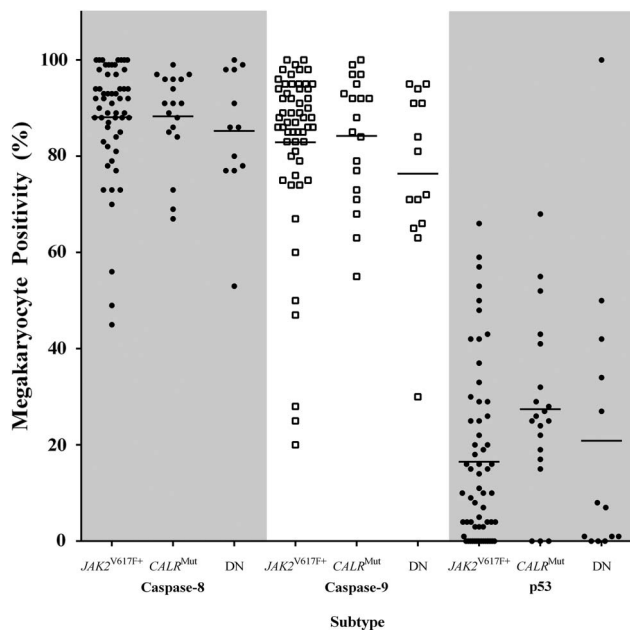


Figure 5 Caspase-8, caspase-9 and p53 in myeloproliferative neoplasms by $JAK2^{V617F}$ and $CALR$ mutation status. The percentage of caspase-8-positive and caspase-9-positive megakaryocytes were similar for $JAK2^{V617F+}$ and $CALR^{Mut}$ and not significantly different in comparison to $JAK2^{V617F+}/CALR^{WT}$ cases, $p>0.05$. p53 megakaryocyte positivity was greater in $CALR^{Mut}$ than $JAK2^{V617F+}$, $p=0.067$. DN—double negative (ie, $JAK2^{V617F-}/CALR^{WT}$).

Diablo-positive megakaryocytes in the MPNs correlates with lower concentrations of the IAP-inhibitor in comparison to stronger survivin signals. This was not seen in control megakaryocytes, where the staining intensity of survivin and Diablo was similar. The survival advantage therefore persists in myeloproliferative megakaryocytes, and in conjunction with other intrinsic apoptotic disturbances is likely to promote their accumulation.

Our data indicate that myeloproliferative megakaryocytes attempt to counteract these survival effects by stimulating the extrinsic apoptotic cascade via caspase-8. This may represent a protective, death-promoting response against megakaryocyte oncogenesis following the accumulation of excess molecular abnormalities. Myeloproliferative megakaryocytes also increase their nuclear p53 content relative to controls where p53 was, and has previously been shown to be, mostly absent.⁴⁹ We postulate that caspase-8 overactivity induces *TP53* gene transcription to produce p53.^{44–45} p53 then translocates to the megakaryocyte nucleus where it activates the transcription of pro-death biomarkers (eg, Fas, Apaf-1) to stimulate both extrinsic and intrinsic apoptotic cascades.⁵⁹ Moreover, megakaryocytic p53 is potentially increased to directly abrogate the antideath effects of survivin.⁴⁸ Alternatively, this p53 upregulation in myeloproliferative megakaryocytes may be to regulate their polyploidisation by inducing states of megakaryocyte senescence.⁶⁰

There were differences in the megakaryocyte apoptotic profiles according to mutation status. Most notably, *CALR*-mutated cases showed greater megakaryocytic p53, but not caspase-8 positivity, than those with the $JAK2^{V617F}$ mutation, although the former was of borderline insignificance. It has previously been shown that calreticulin is involved in caspase-8 activation.²¹ Our data suggest that the mutated calreticulin gene product does not influence caspase-8 levels. Rather, it appears that *CALR* lesions disrupt alternative apoptotic effectors and that affected megakaryocytes will attempt a remedial pro-death response dominated by p53 overexpression. We also show that the small increase in the proportion of caspase-9-positive megakaryocytes in the MPNs is positively correlated with platelet count, and that this correlation strengthens with *CALR* mutations. We propose two explanations for this data. First, it is likely that *CALR* lesions interfere indirectly with caspase-9, rather than with caspase-8. Second, the minor increase in caspase-9 is unlikely to enable megakaryocyte apoptosis to be completed via the intrinsic apoptotic pathway. Rather, several groups have shown that constitutively activated caspase-9 in the presence of intrinsic antiapoptotic effectors is necessary for proplatelet synthesis and shedding.^{24–61–63} Therefore, low-level caspase-9 upregulation and concurrent survivin overexpression, especially among *CALR*-mutated MPNs, may drive thrombocytosis instead of facilitating megakaryocyte apoptosis.

As MPNs progress towards MF, megakaryocytes accumulate additional morphological, topographical and molecular abnormalities, and show the greatest survival signature.^{7–13–64} The megakaryocytes in MF also have greater apoptotic disturbances than the other MPNs. This includes lower caspase-9 and greater numbers of p53-positive megakaryocytes than both PV and ET. It appears that the megakaryocyte attempts to rectify this apoptotic disruption by reducing survivin and overactivating the 'p53-mediated' transcription of its pro-death effectors required for apoptotic execution.⁵⁹ This is consistent with the morphological appearances of megakaryocytes in MF with their greater nuclear pleiomorphism and para-apoptotic changes.^{7–13–65} Further, the reduction in number of caspase-9-positive megakaryocytes in MF may be the

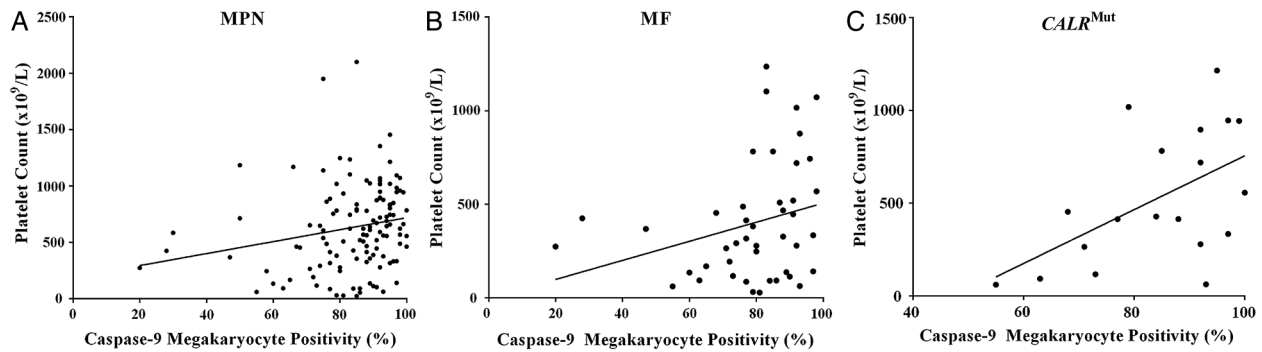


Figure 6 Caspase-9 megakaryocyte positivity correlated significantly with platelet counts for (A) all myeloproliferative neoplasm cases ($r=0.28$, $p=0.0018$), (B) myelofibrotic entities ($r=0.34$, $p=0.026$) and (C) *CALR*-mutated trephines ($r=0.50$, $p=0.030$).

mechanism for the lower platelet counts characteristic of this phenotype compared with other MPN entities.^{7 66}

In summary, disruptions targeting the intrinsic apoptotic cascade appear to promote megakaryocyte accumulation and thrombocytosis in the MPNs. Survivin seems to be a key mediator of this antiapoptotic signature and therefore could be a potential therapeutic target. Anti-survivin agents have been developed, which show therapeutic efficacy for many other malignancies.^{67–69} As such, targeting survivin in the MPNs could potentially control megakaryocyte accumulation and downstream effects (eg, stromal accumulation). Further, a synergistic option could potentially be achieved by including Diablo/Smac-like mimetics^{70–72} to augment innate and already upregulated megakaryocytic Diablo in the MPNs.

Take home messages

- ▶ Dysregulation of intrinsic apoptotic mechanisms in megakaryocytes in the myeloproliferative neoplasms (MPNs) promotes their hyperplasia and thrombocytosis.
- ▶ Survivin, a protein that inhibits the intrinsic pathway of apoptosis, appears to be a key mediator of apoptotic failure of megakaryocytes in the MPNs.
- ▶ Caspase-9 dysfunction may explain the heightened thrombocytosis in MPNs, especially among those with *CALR* mutations.

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Contributors JAJM, KAF and BM performed the immunohistochemical studies. C-CS, H-WI and BGG conducted the molecular analyses. JAJM, KAF, SK, BM and WNE performed the megakaryocyte enumeration for all cases. JAJM conducted the statistical analyses. C-CS, HWI, CF and WNE undertook the morphological review. RH and WNE initiated the study. All authors contributed to the writing of the manuscript.

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Patient consent Obtained.

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Data sharing statement Data are available upon request and at the discretion of the authors.

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REFERENCES

- 1 James C, Ugo V, Le Couédic JP, *et al*. A unique clonal *JAK2* mutation leading to constitutive signalling causes polycythaemia vera. *Nature* 2005;434:1144–8.
- 2 Levine RL, Wadleigh M, Cools J, *et al*. Activating mutation in the tyrosine kinase *JAK2* in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell* 2005;7:387–97.
- 3 Baxter EJ, Scott LM, Campbell PJ, *et al*. Acquired mutation of the tyrosine kinase *JAK2* in human myeloproliferative disorders. *Lancet* 2005;365:1054–61.
- 4 Klampfl T, Gisslinger H, Harutyunyan AS, *et al*. Somatic mutations of calreticulin in myeloproliferative neoplasms. *N Engl J Med* 2013;369:2379–90.
- 5 Nangalia J, Massie CE, Baxter EJ, *et al*. Somatic *CALR* mutations in myeloproliferative neoplasms with nonmutated *JAK2*. *N Engl J Med* 2013;369:2391–405.
- 6 Tefferi A, Thiele J, Orazi A, *et al*. Proposals and rationale for revision of the World Health Organization diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis: recommendations from an *ad hoc* international expert panel. *Blood* 2007;110:1092–7.
- 7 Swerdlow SH, Campo E, Harris NL, *et al*. *WHO classification of tumours, Volume 2*. 4th edn. Geneva: WHO, 2008; WHO classification of tumours of haematopoietic and lymphoid tissues.
- 8 Tefferi A, Vainchenker W. Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. *J Clin Oncol* 2011;29:573–82.
- 9 Florena AM, Tripodo C, Di Bernardo A, *et al*. Different immunophenotypical apoptotic profiles characterise megakaryocytes of essential thrombocythemia and primary myelofibrosis. *J Clin Pathol* 2009;62:331–8.
- 10 Tognon R, Gasparotto EPL, Leroy JMG, *et al*. Differential expression of apoptosis-related genes from death receptor pathway in chronic myeloproliferative diseases. *J Clin Pathol* 2011;64:75–82.
- 11 Treliński J, Chojnowski K, Cebula-Obzut B, *et al*. Impaired apoptosis of megakaryocytes and bone marrow mononuclear cells in essential thrombocythemia: correlation with *JAK2V617F* mutational status and cytoreductive therapy. *Med Oncol* 2012;29:2388–95.
- 12 Koopmans SM, Schouten HC, van Marion AM. Anti-apoptotic pathways in bone marrow and megakaryocytes in myeloproliferative neoplasia. *Pathobiology* 2014;81:60–8.
- 13 Malherbe JAJ, Fuller KA, Arshad A, *et al*. Megakaryocytic hyperplasia is driven by disordered proliferative, apoptotic and epigenetic mechanisms. *J Clin Path* 2016;62:155–63.
- 14 Ghavami S, Hashemi M, Ande SR, *et al*. Apoptosis and cancer: mutations within caspase genes. *J Med Genet* 2009;46:497–510.
- 15 Olsson M, Zhivotovskiy B. Caspases and cancer. *Cell Death Differ* 2011;18:1441–9.
- 16 Ouyang L, Shi Z, Zhao S, *et al*. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferat* 2012;45:487–98.

- 17 Hakem A, El Ghamrasni S, Maire G, *et al.* Caspase-8 is essential for maintaining chromosomal stability and suppressing B-cell lymphomagenesis. *Blood* 2012;119:3495–502.
- 18 Liu JR, Opipari AW, Tan L, *et al.* Dysfunctional apoptosome activation in ovarian cancer: implications for chemoresistance. *Cancer Res* 2002;62:924–31.
- 19 Park JY, Park JM, Jang JS, *et al.* Caspase 9 promoter polymorphisms and risk of primary lung cancer. *Hum Mol Genet* 2006;15:1963–71.
- 20 Gangwar R, Mandhani A, Mittal RD. Caspase 9 and caspase 8 gene polymorphisms and susceptibility to bladder cancer in north Indian population. *Ann Surg Oncol* 2009;16:2028–34.
- 21 Wang W, Groenendyk J, Michalak M. Calreticulin signaling in health and disease. *Int J Biochem Cell B* 2012;44:842–6.
- 22 Vannucchi AM, Rotunno G, Bartalucci N, *et al.* Calreticulin mutation-specific immunostaining in myeloproliferative neoplasms: pathogenetic insight and diagnostic value. *Leukemia* 2014;28:1181–8.
- 23 Stein H, Bob R, Dürkop H, *et al.* A new monoclonal antibody (CAL2) detects CALRETICULIN mutations in formalin-fixed and paraffin-embedded bone marrow biopsies. *Leukaemia* 2016;30:131–5.
- 24 De Botton S, Sabri S, Daugas E, *et al.* Platelet formation is the consequence of caspase activation within megakaryocytes. *Blood* 2002;100:1310–17.
- 25 White MJ, Schoenwaelder SM, Josefsson EC, *et al.* Caspase-9 mediates the apoptotic death of megakaryocytes and platelets, but is dispensable for their generation and function. *Blood* 2012;119:4283–90.
- 26 Chai J, Du C, Wu JW, *et al.* Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* 2000;406:855–62.
- 27 Dohi T, Beltrami E, Wall NR, *et al.* Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis. *J Clin Invest* 2004;114:1117–27.
- 28 Sah NK, Khan Z, Khan GJ, *et al.* Structural, functional and therapeutic biology of survivin. *Cancer Lett* 2006;244:164–71.
- 29 Duffy MJ, O'Donovan N, Brennan DJ, *et al.* Survivin: a promising tumor biomarker. *Cancer Lett* 2007;249:49–60.
- 30 Ren Y, Akyurek N, Schlette E, *et al.* Expression of Smac/DIABLO in B-cell non-Hodgkin and Hodgkin lymphomas. *Hum Pathol* 2006;37:1407–13.
- 31 Pluta A, Wrzesien-Kus A, Cebula-Obrzut B, *et al.* Influence of high expression of Smac/DIABLO protein on the clinical outcome in acute myeloid leukemia patients. *Leuk Res* 2010;34:1308–13.
- 32 Mansour A, Nabil M, Ali-Labib R, *et al.* Reciprocal expression of survivin and SMAC/DIABLO in primary breast cancer. *Med Oncol* 2012;29:2535–42.
- 33 Fulda S. Inhibitor of apoptosis (IAP) proteins in haematological malignancies: molecular mechanisms and therapeutic opportunities. *Leukemia* 2014;28:1414–22.
- 34 Shinozawa I, Inokuchi K, Wakabayashi I, *et al.* Disturbed expression of the anti-apoptosis gene, survivin, and EPR-1 in hematological malignancies. *Leukemia Res* 2000;24:965–70.
- 35 Mori A, Wada H, Nishimura Y, *et al.* Expression of the antiapoptosis gene survivin in human leukemia. *Int J Hematol* 2002;75:161–5.
- 36 Nakagawa Y, Yamaguchi S, Hasegawa M, *et al.* Differential expression of survivin in bone marrow cells from patients with acute lymphocytic leukemia and chronic lymphocytic leukemia. *Leukemia Res* 2004;28:487–94.
- 37 Small S, Keerthivasan G, Huang Z, *et al.* Overexpression of survivin initiates hematologic malignancies *in vivo*. *Leukemia* 2010;24:1920–6.
- 38 Adida C, Haioun C, Gaulard P, *et al.* Prognostic significance of survivin expression in diffuse large B-cell lymphomas. *Blood* 2000;96:1921–5.
- 39 Ansell SM, Arendt BK, Grote DM, *et al.* Inhibition of survivin expression suppresses the growth of aggressive non-Hodgkin's lymphoma. *Leukemia* 2004;18:616–23.
- 40 Martinez A, Bellosillo B, Bosch F, *et al.* Nuclear survivin expression in mantle cell lymphoma is associated with cell proliferation and survival. *Am J Pathol* 2004;164:501–10.
- 41 Schlette EJ, Medeiros LJ, Goy A, *et al.* Survivin expression predicts poorer prognosis in anaplastic large-cell lymphoma. *J Clin Oncol* 2004;22:1682–8.
- 42 Wen Q, Leung C, Huang Z, *et al.* Survivin is not required for the endomitotic cell cycle of megakaryocytes. *Blood* 2009;114:153–6.
- 43 McCrann DJ, Ravid K. Survivin localization during endomitosis of high ploidy mouse megakaryocytes. *Blood* 2010;116:2192–3.
- 44 Schuler M, Bossy-Wetzel E, Goldstein JC, *et al.* p53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. *J Biol Chem* 2000;275:7337–42.
- 45 Chipuk JE, Green DR. Dissecting p53-dependent apoptosis. *Cell Death Differ* 2006;13:994–1002.
- 46 Tomita Y, Marchenko N, Erster S, *et al.* WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. *J Biol Chem* 2006;281:8600–6.
- 47 Deng X, Gao F, Flagg T, *et al.* Bcl2's flexible loop domain regulates p53 binding and survival. *Mol Cell Biol* 2006;26:4421–34.
- 48 Mirza A, McGuirk M, Hockenbery TN, *et al.* Human survivin is negatively regulated by wild type p53 and participate in p53-dependent apoptotic pathway. *Oncogene* 2002;21:2613–22.
- 49 Datta NS, Long MW. Modulation of MDM2/p53 and cyclin-activating kinase during the megakaryocyte differentiation of human erythroleukemia cells. *Exp Hematol* 2002;30:158–65.
- 50 Horie K, Kubo K, Yonezawa M. p53 dependency of radioadaptive responses in spleen colonies and peripheral blood-cell counts in C57BL mice. *J Radiat Res* 2002;43:353–60.
- 51 Mahdi T, Brizard A, Millet C, *et al.* In vitro p53 and/or Rb antisense oligonucleotide treatment in association with growth factors induces the proliferation of peripheral hematopoietic progenitors. *J Cell Sci* 1995;108(Pt 3):1287–93.
- 52 Ritchie A, Vadhan-Raj S, Broxmeyer HE. Thrombopoietin suppresses apoptosis and behaves as a survival factor for the human growth factor-dependent cell line, M07e. *Stem Cells* 1996;14:330–6.
- 53 Apostolidis PA, Woulfe DS, Chavez M, *et al.* Role of tumor suppressor p53 in megakaryopoiesis and platelet function. *Exp Hematol* 2012;40:131–42.
- 54 Apostolidis PA, Lindsey S, Miller WM, *et al.* Proposed megakaryocytic regulon of p53: the genes engaged to control cell cycle and apoptosis during megakaryocytic differentiation. *Physiol Genomics* 2012;44:638–50.
- 55 Harutyunyan A, Klampfl T, Cazzola M, *et al.* p53 lesions in leukemic transformation. *N Engl J Med* 2011;364:488–90.
- 56 Raza S, Viswanatha D, Frederick L, *et al.* TP53 mutations and polymorphisms in primary myelofibrosis. *Am J Hematol* 2012;87:204–6.
- 57 Greaves WO, Verma S, Bisrat T, *et al.* TP53 mutation is rare in primary myelofibrosis. *Leuk Lymphoma* 2013;54:1552.
- 58 Mazur EM, Lindquist DL, de Alarcon PA, *et al.* Evaluation of bone marrow megakaryocyte ploidy distributions in persons with normal and abnormal platelet counts. *J Clin Lab Med* 1988;111:194–202.
- 59 Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Co* 2005;331:851–8.
- 60 Fuhrken PG, Apostolidis PA, Lindsey S, *et al.* Tumor suppressor protein p53 regulates megakaryocytic polyploidization and apoptosis. *J Biol Chem* 2008;283:15589–600.
- 61 White MJ, Kile BT. Apoptotic processes in megakaryocytes and platelets. *Semin Hematol* 2010;47:227–34.
- 62 Josefsson EC, James C, Henley KJ, *et al.* Megakaryocytes possess a functional intrinsic apoptosis pathway that must be restrained to survive and produce platelets. *J Exp Med* 2011;208:2017–31.
- 63 Debrincat MA, Josefsson EC, James C, *et al.* Mcl-1 and Bcl-xL coordinately regulate megakaryocyte survival. *Blood* 2012;119:5850–8.
- 64 Barosi G, Rosti V, Bonetti E, *et al.* Evidence that pre-fibrotic myelofibrosis is aligned along a clinical and biological continuum featuring primary myelofibrosis. *PLoS ONE* 2012;7:e35631.
- 65 Asher E, Payne CM, Berstein C. Evaluation of cell death in EBV-transformed lymphocytes using agarose gel electrophoresis, light microscopy and electron microscopy: II. Induction of non-classic apoptosis ("para-apoptosis") by tritiated thymidine. *Leuk Lymphoma* 1995;19:107–19.
- 66 Gangat N, Caramazza D, Vaidya R, *et al.* DIPSS plus: a refined dynamic international prognostic scoring system for primary myelofibrosis that incorporates prognostic information from karyotype, platelet count and transfusion status. *J Clin Oncol* 2011;29:392–7.
- 67 Weiss A, Brill B, Borghouts C, *et al.* Survivin inhibition by an interacting recombinant peptide, derived from the human ferritin heavy chain, impedes tumor cell growth. *J Cancer Res Clin Oncol* 2012;138:1205–20.
- 68 Groner B, Weiss A. Targeting survivin in cancer: novel drug development approaches. *BioDrugs* 2014;28:27–39.
- 69 Mehta A, Zhang L, Boufraqueh M, *et al.* Inhibition of survivin with YM155 induces durable tumour response in anaplastic thyroid cancer. *Cancer Clin Res* 2015;21:4213–32.
- 70 Runckel KL, Skitzki J, Hernandez F, *et al.* The SMAC mimetic Lcl-161 augments the *in vitro* and *in vivo* anti-tumour activity of rituximab and chemotherapy in rituximab relapse/refractory lymphoma models. *Blood* 2014;124:3110.
- 71 Chromik J, Safferthal C, Serve H, *et al.* Smac mimetic primes apoptosis-resistant acute myeloid leukaemia cells for cytarabine-induced cell death by triggering necroptosis. *Cancer Lett* 2014;344:101–9.
- 72 Amaravadi RK, Schilder RJ, Martin LP, *et al.* A phase I study of the SMAC-mimetic birinapant in adults with refractory solid tumors or lymphoma. *Molec Cancer Ther* 2015;14:2569–75.