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Calpain 2 Activation of P-TEFb Drives Megakaryocyte Morphogenesis and Is Disrupted by Leukemogenic GATA1 Mutation

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SUMMARY

Megakaryocyte morphogenesis employs a "hypertrophy-like" developmental program that is dependent on P-TEFb kinase activation and cytoskeletal remodeling. P-TEFb activation classically occurs by a feedback-regulated process of signal-induced, reversible release of active Cdk9-cyclin T modules from large, inactive 7SK small nuclear ribonucleoprotein particle (snRNP) complexes. Here, we have identified an alternative pathway of irreversible P-TEFb activation in megakaryopoiesis that is mediated by dissolution of the 7SK snRNP complex. In this pathway, calpain 2 cleavage of the core 7SK snRNP component MePCE promoted P-TEFb release and consequent upregulation of a cohort of cytoskeleton remodeling factors, including α-actinin-1. In a subset of human megakaryocytic leukemias, the transcription factor GATA1 undergoes truncating mutation (GATA1s). Here, we linked the GATA1s mutation to defects in megakaryocytic upregulation of calpain 2 and of P-TEFb-dependent cytoskeletal remodeling factors. Restoring calpain 2 expression in GATA1s mutant megakaryocytes rescued normal development, implicating this morphogenetic pathway as a target in human leukemogenesis.

INTRODUCTION

Mammalian hematopoietic differentiation proceeds by a series of binary decisions that yield progenitors of increasingly limited developmental potential, with the megakaryocyte lineage emerging from a bipotent megakaryocyte-erythroid progenitor (MEP). Megakaryocytic and erythroid cells, despite common origins, shared transcription factors, and shared signaling pathways, differ profoundly in their developmental programs. Erythroid morphogenesis occurs through progressive reduction in cell size accompanied by nuclear condensation and ultimately extrusion. Megakaryocytic morphogenesis contrastingly involves marked expansion in cell mass combined with acquisition of a lobulated, polyploid nucleus containing up to *32-64N*. In this regard, megakaryopoiesis bears resemblance to the program of cardiomyocyte hypertrophy, in which pressure overload elicits cellular enlargement and polyploidization (Liu et al., 2010).

Mechanistically, the developmental morphogenesis of megakaryocytes and the hypertrophic response of cardiomyocytes share key regulatory elements. At the transcriptional level, both programs rely on a complex of serum response factor (SRF) with myocardin-related transcription factors (Cheng et al., 2009; Halene et al., 2010; Kuwahara et al., 2010; Nelson et al., 2005; Smith et al., 2012), as well as on MEF2C (Gekas et al., 2009; Muñoz et al., 2009). At the signaling level, both programs require high-amplitude activation of the P-TEFb kinase pathway (Elagib et al., 2008; Sano et al., 2002). A critical distinction is that cardiomyocyte hypertrophy consists of a reversible response to a pathologic stimulus, whereas normal megakaryocyte morphogenesis represents an irreversible, terminal program largely driven by cell-intrinsic mechanisms.

Cellular P-TEFb kinase activity is tightly regulated, with the majority of the Cdk9-cyclin T kinase modules sequestered in large inactive complexes containing the 7SK snRNA, HEXIM1, MePCE, LARP7, and additional factors (Barboric et al., 2009; Jeronimo et al., 2007; Peterlin and Price, 2006; Price, 2008; Xue et al., 2010). Several stimuli, including hypertrophic agonists (e.g., endothelin-1), UV irradiation, HIV-1 Tat, and hexamethylene bisacetamide activate P-TEFb by inducing the release of Cdk9-cyclin T from the 7SK small nuclear ribonucleoprotein particle (snRNP) complex (Chen et al., 2008; Contreras et al., 2007; Krueger et al., 2010; Sano et al., 2002; Sano and Schneider, 2004). These stimuli variously trigger signaling via Gq, calcineurin, PP1α, and PI3K, ultimately leading to remodeling of the 7SK snRNP that promotes dissociation of Cdk9-cyclin T and HEXIM1 away from the stable core complex of 7SK, MePCE, and LARP7. Once activated, P-TEFb promotes transcriptional elongation by phosphorylating RNA polymerase II and associated pausing factors. Feedback autoregulation results from the rapid and potent induction of *HEXIM1* transcription

(Bartholomeeusen et al., 2012; Garriga et al., 2010; He et al., 2006), effectively driving resequestration of Cdk9-cyclin T back into an inactive 7SK snRNP complex (Bartholomeeusen et al., 2012; Zhou et al., 2012).

GATA1, a master transcriptional regulator of megakaryocyte and erythroid differentiation, physically and functionally interacts with P-TEFb in hematopoietic cells (Bottardi et al., 2011; Elagib et al., 2008). Somatic mutations yielding an N-terminal truncated, "short" GATA1 protein (GATA1s) occur in virtually all megakaryocytic neoplasms associated with Down syndrome (Wickrema and Crispino, 2007). In knockin mice, the mutant GATA1s induces transient megakaryocytic hyperproliferation and maturational defects during fetal liver hematopoiesis (Li et al., 2005). Megakaryocytic hyperproliferation and aberrant differentiation have also been elicited by P-TEFb inhibition in adult mice with megakaryocytic GATA1 deficiency, supporting the notion of a GATA1-P-TEFb megakaryopoietic pathway that might be affected in Down syndrome neoplasms (Elagib et al., 2008).

In the current study, we have identified a megakaryopoietic P-TEFb activation pathway characterized by downregulation of the 7SK snRNP core components MePCE, LARP7, and 7SK snRNA. The protease calpain 2 critically participated in this pathway, undergoing recruitment to P-TEFb, targeting MePCE for proteolysis, and promoting P-TEFb-dependent megakaryocyte morphogenesis. A cohort of coregulated cytoskeletal remodeling factors involved in execution of the morphogenetic program was identified in this pathway downstream of P-TEFb. In a large panel of human megakaryocytic leukemias, decreased calpain 2 levels significantly correlated with the presence of the GATA1s mutation. In addition, murine fetal liver megakaryocytes from GATA1s knockin mice displayed defects in upregulation of calpain 2 and of downstream cytoskeletal remodeling factors. Lentiviral restoration of calpain 2 expression specifically ameliorated developmental defects in GATA1s knockin fetal megakaryocytes. These findings thus support a megakaryocyte morphogenetic pathway involving GATA1, calpain 2, P-TEFb, and the actin cytoskeleton. Perturbations of this pathway may play a role in the pathogenesis of Down syndrome megakaryocytic neoplasms.

RESULTS

Global P-TEFb Activation in Megakaryopoiesis

Previous work has suggested a critical role for high-amplitude P-TEFb activation in megakaryocyte differentiation and divergence from the erythroid lineage (Elagib et al., 2008). To examine the mechanistic basis for this activation, 7SK snRNP complex components were quantified in megakaryocytic, erythroid, and undifferentiated cells derived from primary human hematopoietic progenitors. The principal P-TEFb factors in hematopoietic cells, Cdk9 and cyclin T1, showed similar protein levels in megakaryocytic (Mk), undifferentiated (Un), and erythroid (Ery) cells (Figure 1A). By contrast, megakaryocytic cells specifically downregulated all of the components of the recently defined (Barboric et al., 2009; Xue et al., 2010) 7SK snRNP core complex: MePCE (Me), LARP7 (L7), and the 7SK snRNA (Figures 1A and 1B). Additionally, megakaryocytic cells displayed enhanced phosphorylation of RNA polymerase II carboxy terminal domain serine 2 (RNAPII S2), a specific target of P-TEFb phosphorylation (Peterlin and Price, 2006) (Figure 1C). Concomitant with downregulation of the 7SK inhibitory scaffold, megakaryocytes specifically upregulated HEXIM1, reflecting increased cellular P-TEFb activity (Bartholomeeusen et al., 2012; Garriga et al., 2010; He et al., 2006) (Figure 1A). The megakaryocytic induction of HEXIM1 occurred at the mRNA level (Figure S1A available online) and was prevented by the Cdk9 inhibitor flavopiridol (FP) and by shRNA knockdown of Cdk9 (Figure S1B). *MEPCE* mRNA levels showed no significant decline during megakaryocytic differentiation, suggesting regulation of this factor at the protein level (Figure S1C).

Similar studies were carried out on a nontransformed murine hematopoietic cell line, HPC7, which retains cytokine-responsive multilineage differentiation potential (Pinto do O et al., 1998). These cells underwent rapid and efficient erythroid or megakaryocytic differentiation in response to 48 hr treatment with erythropoietin (Ery) or thrombopoietin (Mk), respectively (Figure S1D). As with primary human progenitors, HPC7 megakaryocytic differentiation specifically correlated with downregulation of the 7SK snRNP core components and upregulation of HEXIM1 (Figures 1D, 1E, and S1E). In addition, the HPC7 cells did not downregulate cyclin T1 or Cdk9 during megakaryocytic differentiation (Figure S1F). Glycerol gradient analysis of Cdk9 distribution between large, inactive and small, active complexes (Sedore et al., 2007) revealed 80% of megakaryocytic Cdk9 to be within the small complex (fraction 5). In erythroid and undifferentiated cells, by contrast, the majority of Cdk9 (~70%) resided in the large complex (fraction 9) (Figure 1F). (Fraction 3 contained insoluble debris and represents background.) Another validated approach toward assessment of intracellular P-TEFb status consists of immunoprecipitation to determine association with the repressor HEXIM1, an interaction dependent on the integrity of the 7SK snRNP core complex (Chen et al., 2008). This approach confirmed a decrease in cyclin T1-HEXIM1 complexes in HPC7 cells undergoing megakaryocytic differentiation, as compared with cells either undergoing erythroid differentiation (Figure 1G) or maintained undifferentiated (see Figure 2C). The results in Figure 1 thus suggest a lineage-specific mechanism of P-TEFb activation in megakaryopoiesis, during which the dissolution of the 7SK snRNP is accompanied by large-scale release of active P-TEFb.

Calpain Contribution to Megakaryocytic P-TEFb Activation and Differentiation

Mapping of the mammalian transcriptional protein interactome has identified calpain subunits (S1 and 2) as components of an extended 7SK snRNP network (Jeronimo et al., 2007), suggesting a role for proteolysis in remodeling of this complex. To examine the association of calpain with P-TEFb in a cell line model of megakaryocyte differentiation, K562 cells before and after phorbol ester (TPA) treatment underwent immunoprecipitation of endogenous cyclin T1 (CT1). As described for GATA1 (Elagib et al., 2008), calpain 2 displayed inducible recruitment to P-TEFb in association with differentiation induction (Figure 2A). By contrast, Cdk9 coprecipitated with cyclin T1 both before and after induction. Immunoprecipitation of endogenous HEXIM1 also revealed calpain 2 binding that was enhanced by TPA treatment, suggesting inducible recruitment of calpain 2 to the 7SK snRNP complex during megakaryocytic differentiation (Figure S2A).