HEXIM1 induction is mechanistically involved in mediating anti-AML activity of BET protein bromodomain antagonist

Santhana G. T. Devaraj[1], **Warren Fiskus**[2], **Bhavin Shah**[1], **Jun Qi**[3], **Baohua Sun**[1], **Swaminathan P Iyer**[1], **Sunil Sharma**[4], **James E. Bradner**[3], and **Kapil N. Bhalla**[5]

[1]Houston Methodist Research Institute, Houston, TX, 77030

[2]Baylor College of Medicine, Houston, TX, 77030

[3]Dana-Farber Cancer Institute, Boston, MA, 0221

[4]University of Utah, Huntsman Cancer Institute; Salt Lake City, 84112

[5] M.D. Anderson Cancer Center, Houston, TX, 77030

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BET protein bromodomain antagonists (BA) have been shown to inhibit growth, induce apoptosis and exert promising anti-AML activity, especially against those AML cells that display genetic mutation in NPM1, MLL, FLT3, IDH2 and EVI1 ([1]–[6]). BAs, such as the prototype JQ1, disrupt the binding of the BET proteins, e.g., BRD4 to acetylated histones ([7], [8]). This inhibits the BRD4-mediated recruitment of the positive transcription elongation factor b (pTEFb), a heterodimer of CDK9 and Cyclin T1, for inducing RNA polymerase-II (RNAP2) phosphorylation ([7], [8]). pTEFb phosphorylates serine-2 in the C-terminal domain heptad repeats $Y_1S_2P_3T_4S_5P_6S_7$ of RNAP2, which induces the pause-release of RNAP2, allowing productive mRNA transcript elongation ([8], [9]). The pTEFb-induced phosphorylation events are considered to be rate-limiting for the RNAP2-mediated elongation of mRNA transcripts, including those of several oncogenes such as Myc, BCL2 and CDK4/6 in AML cells ([1], [2], [8]–[10]). Consistent with this, treatment with JQ1 has been shown to reduce the levels of c-Myc, CDK6 and BCL2, associated with growth inhibition and apoptosis of human AML blast progenitor cells (BPCs) ([1], [2], [6]). BA treatment also induces the mRNA and protein expression of hexamethylene bisacetamide-inducible protein 1 (HEXIM1) in AML cells ([6], [8], [11]). HEXIM1 inhibits pTEFb by binding to Cyclin T1 and sequestering pTEFb into an inhibitory complex that also contains the small non-coding RNA 7SK ([8], [11]). HEXIM1 can form homodimers or hetrodimers with the closely related but distinct gene product HEXIM2 ([11], [12]). Multiple pTEFb units bind to a HEXIM1 multimer

Address correspondence to: Kapil N. Bhalla, M.D., Professor, Leukemia Department, M.D. Anderson Cancer Center, 1400 Holcombe Blvd, Unit 428, Houston, Texas 77030-4009, ; Email: kbhalla@mdanderson.org

([12], [13]). By sequestering and inhibiting pTEFb, and in turn RNAP2, HEXIM1 may be mechanistically involved in mediating BA-induced growth inhibition, differentiation and apoptosis of AML cells ([8]–[12]). Interrogation of The Cancer Genome Atlas (TCGA) database utilizing the cBioPortal for Cancer Genomics demonstrated that HEXIM1 mRNA overexpression is almost mutually exclusive with c-Myc overexpression in AML (Fig. 1a) ([14]). Of the 200 AML samples, 22 samples showed c-Myc and 12 samples HEXIM1 overexpression (Fig. 1a). Only one sample showed overexpression of both genes (Fig. 1a). Collectively, these observations created the rationale for further determining the mechanistic role of BA-induced HEXIM1 in mediating the growth inhibition, differentiation and apoptosis of AML BPCs due to treatment with BA.

In the present studies we first knocked down the mRNA and protein expression of HEXIM1 in the cultured AML MOLM13 and patient-derived primary (p) AML BPCs. As compared to the MOLM13 cells transduced with lentivirus-containing, non-targeted shRNA (MOLM13-NT cells), MOLM13-HKD cells transduced with the shRNA specific for HEXIM1 exhibited marked attenuation of the mRNA and protein levels of HEXIM1 (Fig 1b). MOLM13-HKD cells also showed (by confocal immunofluorescence) depletion of the nuclear levels of HEXIM1 (Fig. 1b). Notably, as compared to the MOLM13-NT, MOLM13-HKD cells showed greater increase in cell numbers when placed in suspension culture over 96 hours ($p < 0.05$) (Fig. 1d). Next, we determined the effect of the BA JQ1 on MOLM13-NT versus MOLM13-HKD cells. JQ1 treatment induced significantly more HEXIM1 protein levels in MOLM13-NT versus MOLM13-HKD cells (Fig. 1c). The levels of HEXIM2 were undetectable and were not induced by JQ1 (Fig. 1c). As compared to MOLM13-NT cells, c-Myc expression was higher in the untreated MOLM13-HKD cells, and treatment with JQ1 attenuated c-Myc levels in MOLM13-HKD cells (Fig. 1c). JQ1 treatment was less effective in inhibiting the suspension culture growth of MOLM13-HKD versus MOLM13-NT cells (Fig. 1d). JQ1 induced less morphologic features of differentiation and less % of differentiated MOLM13-HKD cells, as compared to MOLM13-NT cells (Fig. 1e). JQ1 also dose-dependently induced more apoptosis of MOLM13-NT versus MOLM13-HKD cells (Fig. 1f). We also transduced the HEXIM1 shRNA into freshly procured pAML BPCs expressing FLT3-ITD (pAML-HKD cells). As shown in Fig. 1g, compared to the pAML cells transduced with non-targeted shRNA (pAML-NT cells), pAML-HKD cells expressed markedly lower protein levels of HEXIM1 but higher levels of c-Myc, whereas HEXIM2 levels were similar in the two cell types. Notably, treatment with JQ1 induced HEXIM1 in pAML-NT but not in pAML-HKD cells, while HEXIM2 levels remained unperturbed (Fig. 1g). JQ1 treatment also attenuated c-Myc levels (Fig. 1g). Concomitantly, JQ1 treatment induced differentiation in a greater % of pAML-NT BPCs, as compared to pAML-HKD cells (Fig. 1h). Moreover, treatment with JQ1 dose-dependently induced significantly more apoptosis of pAML-NT, as compared to pAML-HKD BPCs (Fig. 1i).

In the nucleus, the regulatory subunit of pTEFb, Cyclin T1, binds to CDK9 or to the C-terminal domain (TBD) of HEXIM1 ([11]), based on which we determined the effect of HEXIM1 knockdown on the binding of Cyclin T1 to HEXIM1 and CDK9 in unperturbed and JQ1 treated MOLM13-NT cells. In the unperturbed MOLM13-NT cells, immunoprecipitated Cyclin T1 bound to HEXIM1 and CDK9 (Supplemental Fig. 1a).

Treatment with JQ1 increased the levels and binding of immunoprecipitated Cyclin T1 to HEXIM1, which was not observed in MOLM13-HKD cells (Supplemental Fig. 1a). JQ1 treatment also caused increased binding of Cyclin T1 to CDK9 in both MOLM13-NT and MOLM13-HKD cells (Supplemental Fig. 1a), without causing increase in the levels of Cyclin T1 and CDK9 (Supplemental Fig. 1b). These results were confirmed by confocal immunofluorescence microscopy, which also demonstrated that the nuclear co-localization of Cyclin T1 and HEXIM1 in MOLM13-NT cells was not observed in MOLM13-HKD cells (Supplemental Fig. 1c). Moreover, increased nuclear co-localization of Cyclin T1 and HEXIM1 in MOLM13-NT cells due to JQ1 treatment was abrogated in MOLM13-HKD cells (Supplemental Fig. 1c).

We next determined the in vivo anti-AML activity of JQ1 against the xenografts of MOLM13-HKD cells compared to the xenografts of MOLM13-NT cells. Two weeks after a tail vein infusion of the MOLM13-NT or MOLM13-HKD cells into the NOD/SCID mice, mice engrafted with the AML cells were treated with either vehicle alone or JQ1 for three weeks. In an additional cohort of mice treated identically with either vehicle alone or JQ1 for 5 days, the bone marrow and spleen were harvested to determine the protein expression of HEXIM1, Cyclin T1, and CDK9. As shown in Fig. 2a, compared to treatment with vehicle alone, JQ1 treatment significantly improved the median survival of the mice engrafted with MOLM13-NT ($p < 0.005$). In contrast, following JQ1 treatment, the improvement in the median survival was significantly compromised in the mice engrafted with MOLM13-HKD cells ($p < 0.01$). Notably, in vivo treatment with JQ1 markedly induced the protein expression of HEXIM1 in the AML cells harvested from the bone marrow of mice engrafted with MOLM13-NT, but not of the MOLM13-HKD cells (Fig. 2b). Following JQ1 treatment, the protein levels of CDK9 were comparable in the engrafted MOLM13-NT and MOLM13-HKD AML cells (Fig. 2b).

We next determined the effects of lentivirus transduced tetraCycline-inducible, ectopic expression of FLAG-tagged HEXIM1 on JQ1-mediated growth inhibition, differentiation and apoptosis of MOLM13 AML cells (MOLM-HIN cells). Fig. 2c demonstrates that, as compared to the untreated cells, exposure of MOLM-HIN cells to 0.5 μg/mL of tetracycline markedly induced the expression of HEXIM1, without affecting the levels of CDK9 or Cyclin T1. This was associated with a marked attenuation of the suspension culture growth of MOLM-HIN cells (Supplemental Fig. 2). Following treatment with JQ1 (100 nM) for 96 hours, as compared to the un-induced cells, tetracycline-induced MOLM-HIN cells demonstrated more profound morphologic features of differentiation (Fig. 2d). Treatment with JQ1 further and significantly increased the % of differentiated MOLM-HIN cells that had been exposed to tetracycline ($p < 0.01$) (Fig. 2e). Notably, treatment with JQ1 for 48 hours also induced significantly more apoptosis of tetracycline-exposed MOLM-HIN cells, as compared to those untreated with tetracycline (Fig. 2f).

The amount and availability of the free and active pTEFb, for mediating RNAP2 pause-release, is negatively controlled by its binding to the ribonucleoprotein complex containing HEXIM1 and snRNA 7SK, a complex in which up to half of the nuclear pTEFb may be sequestered ([8], [9]). This suggests that the nuclear levels of HEXIM1 would influence the growth of the transformed cells by controlling gene expression that regulates cell growth ([8],

[11]). Consistent with the TCGA data, our findings also demonstrate that knockdown of nuclear HEXIM1 is associated with an increase in c-Myc levels. These perturbations in HEXIM1 and c-Myc expressions increased the in vitro growth of the AML cells. However, HEXIM1 knockdown appeared to exert little effect on the in vivo growth of MOLM13-HKD cells, since the survival of the NOD/SCID mice engrafted with MOLM13-HKD cells was similar to those engrafted with MOLM13-NT cells. As noted above, BRD4 may directly recruit pTEFb to activate transcription, or the ET-domain of BRD4 may activate transcription independent of pTEFb by recruiting the arginine demethylase JMJD6 or NSD3 ([8]). This would explain why knockdown of HEXIM1 would not influence all transcription regulated by BRD4.

Collectively, these findings demonstrate that BA-mediated HEXIM1 induction mechanistically regulates BA-induced differentiation and apoptosis of cultured and primary AML BPCs. Further development and in vivo testing of BAs against AML, with careful attention given to HEXIM1 induction as a predictive biomarker of anti-AML activity of BA, is warranted. It would also be important to develop and test other novel agents that induce HEXIM1 for their ability to mediate growth inhibition, differentiation and apoptosis of AML BPCs, especially of the genetic subtypes noted above ([15]).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

REFERENCES

1. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature. 2011; 478:529–533. [PubMed: 21964340]

2. Fiskus W, Sharma S, Qi J, Valenta JA, Schaub LJ, Shah B, et al. Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myelogenous leukemia cells. Mol Cancer Ther. 2014; 13:1142–1154. [PubMed: 24435446]

3. Dawson MA, Gudgin EJ, Horton SJ, Giotopoulos G, Meduri E, Robson S, et al. Recurrent mutations, including NPM1c, activate a BRD4-dependent core transcriptional program in acute myeloid leukemia. Leukemia. 2014; 28:311–320. [PubMed: 24220271]

4. Chen C, Liu Y, Lu C, Cross JR, Morris JPt, Shroff AS, et al. Cancer-associated IDH2 mutants drive an acute myeloid leukemia that is susceptible to Brd4 inhibition. Genes Dev. 2013; 27:1974–1985. [PubMed: 24065765]

5. Groschel S, Sanders MA, Hoogenboezem R, de Wit E, Bouwman BA, Erpelinck C, et al. A single oncogenic enhancer rearrangement causes concomitant EVI1 and GATA2 deregulation in leukemia. Cell. 2014; 157:369–381. [PubMed: 24703711]

6. Fiskus W, Sharma S, Qi J, Shah B, Devaraj SG, Leveque C, et al. BET protein antagonist JQ1 is synergistically lethal with FLT3 tyrosine kinase inhibitor (TKI) and overcomes resistance to FLT3-TKI in AML cells expressing FLT-ITD. Mol Cancer Ther. 2014; 13:2315–2327. [PubMed: 25053825]

7. Belkina AC, Denis GV. BET domain co-regulators in obesity, inflammation and cancer. Nat Rev Cancer. 2012; 12:465–477. [PubMed: 22722403]

8. Shi J, Vakoc CR. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. Mol Cell. 2014; 54:728–736. [PubMed: 24905006]

9. Nechaev S, Adelman K. Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation. Biochimica et biophysica acta. 2011; 1809:34–45. [PubMed: 21081187]

10. Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell. 2013; 153:320–334. [PubMed: 23582323]

11. Liu P, Xiang Y, Fujinaga K, Bartholomeeusen K, Nilson KA, Price DH, et al. Release of positive transcription elongation factor b (P-TEFb) from 7SK small nuclear ribonucleoprotein (snRNP) activates hexamethylene bisacetamide-inducible protein (HEXIM1) transcription. J Biol Chem. 2014; 289:9918–9925. [PubMed: 24515107]

12. Byers SA, Price JP, Cooper JJ, Li Q, Price DH. HEXIM2, a HEXIM1-related protein, regulates positive transcription elongation factor b through association with 7SK. J Biol Chem. 2005; 280:16360–16367. [PubMed: 15713662]

13. Dulac C, Michels AA, Fraldi A, Bonnet F, Nguyen VT, Napolitano G, et al. Transcription-dependent association of multiple positive transcription elongation factor units to a HEXIM multimer. J Biol Chem. 2005; 280:30619–30629. [PubMed: 15994294]

14. The Cancer Genome Atlas Research Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. New Engl J Med. 2013; 368:2059–2074. [PubMed: 23634996]

15. Zhong B, Lama R, Ketchart W, Montano MM, Su B. Lead optimization of HMBA to develop potent HEXIM1 inducers. Bioorg Med Chem Lett. 2014; 245:1410–1413. [PubMed: 24503105]
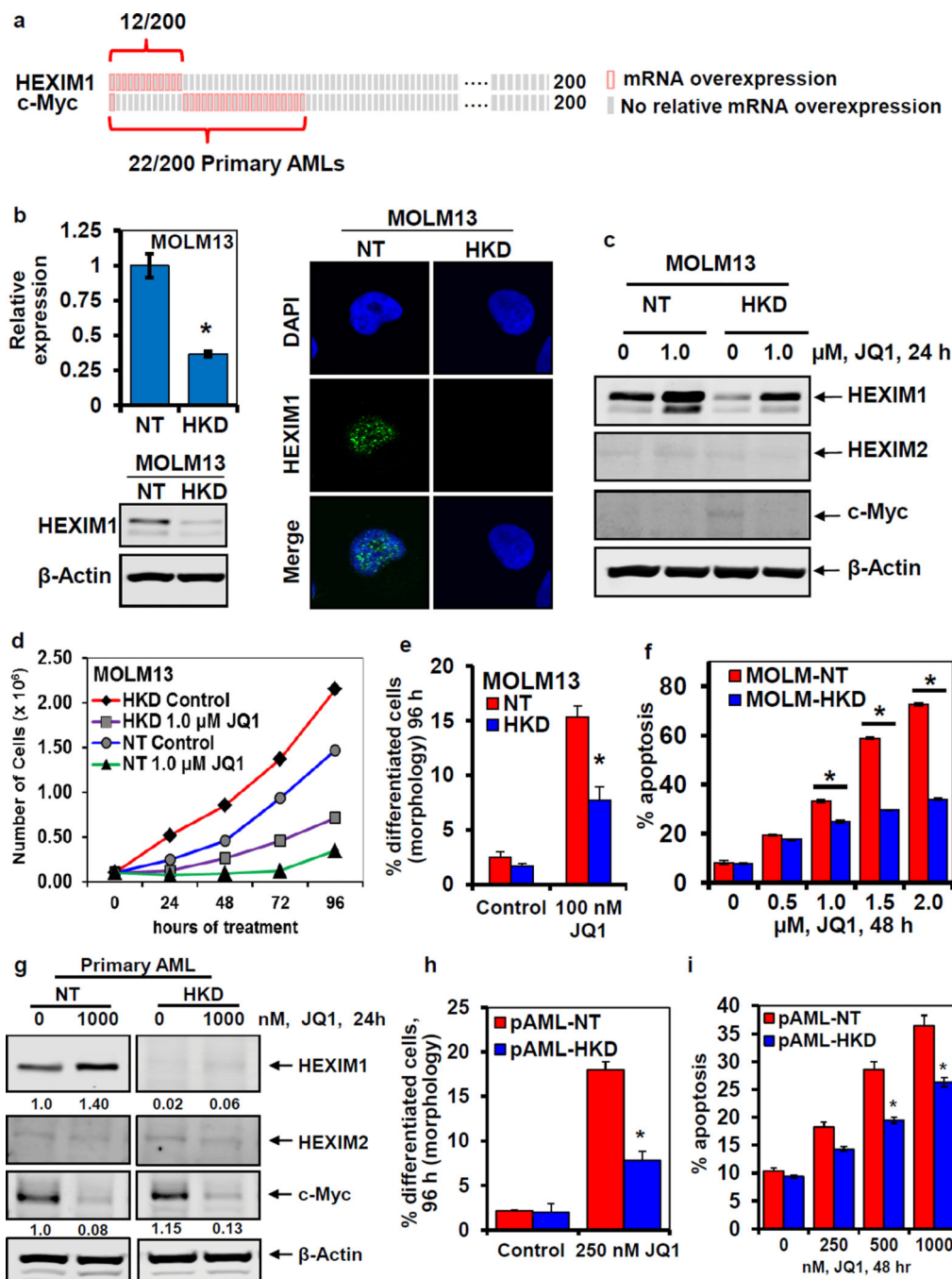
Figure 1. Silencing of HEXIM1 by shRNA attenuates JQ1-mediated induction of HEXIM1, cell differentiation and apoptosis in cultured and primary AML cells

a. Expression status of HEXIM1 and c-Myc in the TCGA AML patient dataset accessed utilizing the cBioPortal (cbioportal.org). An mRNA expression z-score threshold of + 1.5 was utilized for the analysis against the 200 AML samples in this dataset. Red rectangles indicate over expression. Gray rectangles indicate samples with no overexpression of HEXIM1 or c-Myc. **b.** MOLM13 cells were transfected with non-targeting shRNA (sh-NT) and HEXIM1 shRNA (HKD) for 48 hours. Total RNA was isolated and reverse transcribed.

The resulting cDNA was used for real-time, quantitative PCR analysis of HEXIM1. The relative mRNA expression was normalized to GAPDH and compared to the untreated cells. * indicates expression values that are significantly less in MOLM13-HKD cells compared to NT controls. Additionally, total cell lysates were prepared and immunoblot analyses were conducted for the expression of HEXIM1 and β–actin. Transfected cells were also used for immunofluorescence analysis of HEXIM1. Nuclei were stained with DAPI. Images were obtained utilizing confocal microscope equipped with a CCD camera. **c**. MOLM13-NT and MOLM13-HKD cells were treated with JQ1 for 24 hours. Immunoblot analyses were conducted for the expression levels of HEXIM1 HEXIM2, c-Myc and β-Actin in the cell lysates. **d**. MOLM13 sh-NT and MOLM13-HKD cells were plated as indicated (triplicates) and treated with the indicated concentrations of JQ1. Cell counts were obtained every 24 hours of treatment for 96 hours. Values represent the mean of 3 experiments ± S.D. **e**. MOLM13-NT and MOLM13-HKD cells were treated with the indicated concentrations of JQ1 for 96 hours. Following this, cells were cytospun onto glass slides, and stained with hematoxylin and eosin. The % of differentiated cells was determined by light microscopy. Columns, mean of three experiments; Bars, S.E.M. *indicates values that are significantly less in HEXIM1 knockdown (HKD) cells compared to sh-NT cells ($p < 0.05$). **f**. MOLM13 sh-NT and HKD cells were treated with JQ1 for 48 hours. The % apoptotic cells were determined by flow cytometry. Columns, mean of three experiments; Bars, S.E.M. * indicates values that are significantly less in HEXIM1 knockdown (HKD) cells compared to sh-NT cells ($p < 0.05$). **g**. Primary AML cells with sh-NT or sh-HEXIM1 were treated with the indicated concentrations of JQ1 for 24 hours. Immunoblot analyses were conducted as indicated. Numbers beneath the bands represent densitometry analysis performed on representative blots. **h.** MOLM13-NT and MOLM13-HKD cells were treated with the indicated concentrations of JQ1 for 96 hours. Following this, cells were cytospun onto glass slides, and stained with hematoxylin and eosin. The % of differentiated cells was determined by light microscopy. Columns, mean of three experiments; Bars, S.E.M. * indicates values that are significantly less in HEXIM1 knockdown (HKD) cells compared to sh-NT cells ($p < 0.05$). **i.** Primary AML cells with sh-NT or -HKD were treated with the indicated concentrations of JQ1 for 48 hours. The % of apoptotic cells was determined by flow cytometry. Columns, mean of three experiments; Bars, S.E.M. * indicates values that are significantly less in HEXIM1 knockdown (HKD) cells compared to sh-NT cells ($p < 0.05$).
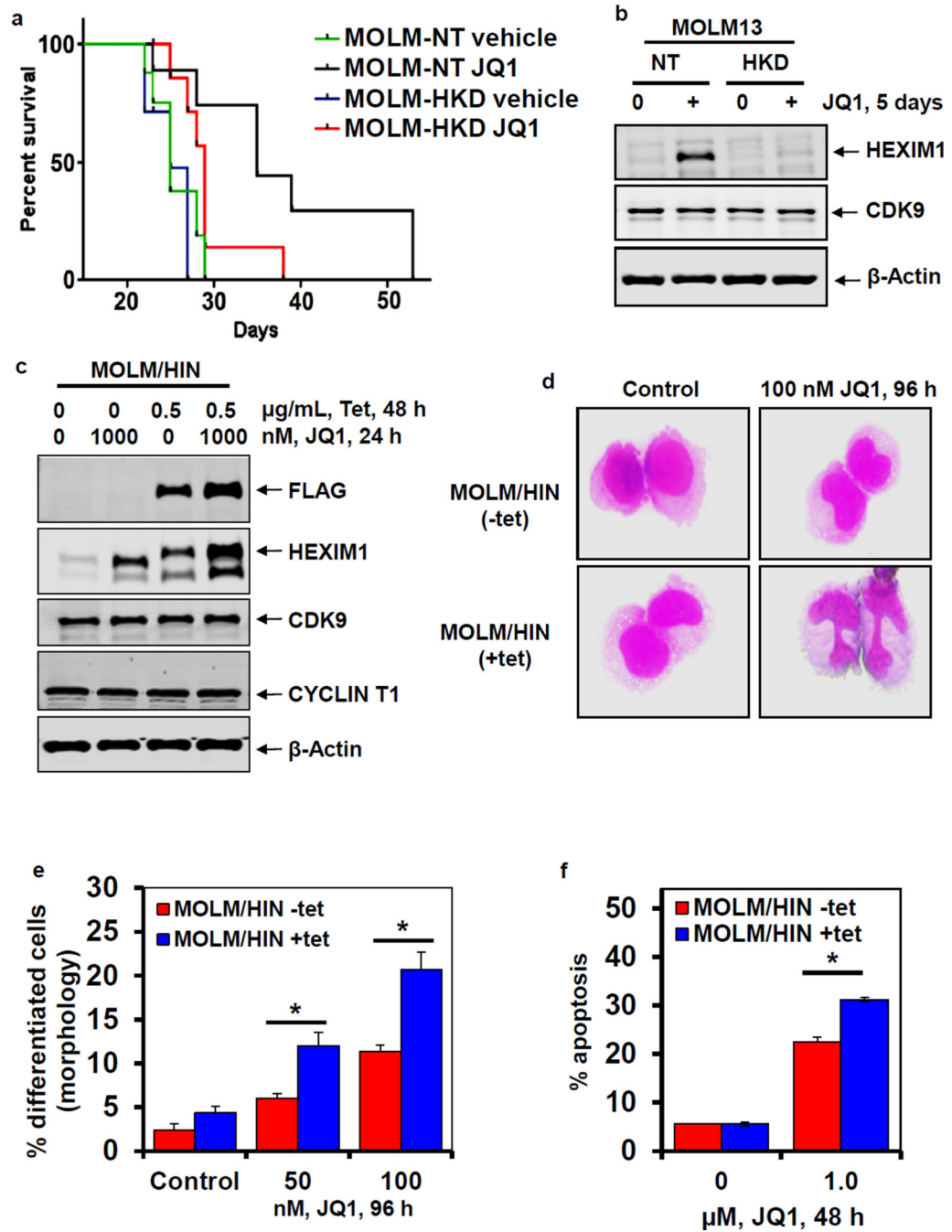
Figure 2. Silencing of HEXIM1 inhibits JQ1-mediated survival benefit in mice bearing AML xenografts whereas tet-inducible HEXIM1 markedly enhances sensitivity to JQ1 mediated differentiation and apoptosis in AML cells

a. MOLM13-NT and MOLM13-HKD were injected into the lateral tail vein of NOD/SCID mice (n=6) that had been pre-conditioned with 2.5 Gy of gamma irradiation. Two weeks post implantation; mice were treated with 50 mg/kg of JQ1 daily (x 5 days) for 3 weeks. Survival of the mice is represented as a Kaplan Meier plot. **b**. In a separate cohort of mice engrafted with MOLM13-NT and MOLM13-HKD cells, mice were treated with 50 mg/kg of JQ1 for 5 days and then sacrificed. Bone marrow was extracted and immunoblot analyses were

conducted as indicated. The expression of β-Actin in the cell lysates served as the loading control. **c.** MOLM13 cells were transfected with a tet-inducible expression vector encoding HEXIM1 (MOLM/HIN). Cells were treated with Tet and JQ1 as indicated. Then, immunoblot analyses were conducted as indicated. **d–e.** MOLM13/HIN cells were treated with or without tetracycline and the indicated concentrations of JQ1 for 96 hours. Then, cells were cytospun onto glass slides, stained with hematoxylin and eosin and the percentage of differentiated cells were determined by light microscopy. Original magnification is 40X. Columns, mean of three experiments; Bars, S.E.M. * indicates values that are significantly greater in HEXIM1-induced cells compared to un-induced cells (p < 0.05). **f.** The % of annexin V-positive apoptotic cells were determined by flow cytometry following Tet induction and treatment with JQ1.