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ABSTRACT

Interferons are useful drugs in treating chronic myeloid leukemia (CML). One of the cellular responses of interferon treatment is the activation of protein modification by ISG15. We have cloned a novel gene encoding a protease UBP43 that specifically removes ISG15 from ISG15 modified proteins. Furthermore, we have generated UBP43 knockout mice. UBP43 deficient hematopoietic cells have much higher levels of ISG15 modified proteins upon interferon stimulation and are hypersensitive to interferon treatment. This grant is to demonstrate that protein ISG15 modification is crucial for interferon function in CML treatment and to analyze the effect of UBP43 on CML development. In the past funding period, we have established BCR/ABL positive leukemia cell lines that have higher than normal or lower than normal level of ISG15 conjugation and initiated the characterization of these cell lines. Furthermore, we have generated additional important data to support that UBP43 knockout mice are resistant to BCR/ABL induced CML development and that interferon plays a critical role in this process. We will continue to perform experiments as proposed in the application to establish the role of ISG15 modification and UBP43 in interferon signaling and CML development.

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Introduction:

Interferons are widely used in the treatment of cancers, especially chronic myeloid leukemia (CML). Although a recently developed new drug - imatinib mesylate (STI571) has shown tremendous success in treating CML, interferons will continually be a crucial player in CML treatment, especially to patients who have developed resistance to imatinib. One of the cellular responses of interferon treatment is the activation of protein modification by ISG15. We have cloned a novel gene encoding a protease UBP43 that specifically removes ISG15 from ISG15 modified proteins. Furthermore, we have generated UBP43 knockout mice. UBP43 deficient hematopoietic cells have much higher levels of ISG15 modified proteins upon interferon stimulation and are hypersensitive to interferon treatment. Most importantly, in contrast to wild type bone marrow cells with BCR/ABL expression that rapidly develop a myeloproliferation disorder resemble human CML, mice transplanted with BCR/ABL expressing UBP43 deficient bone marrow cells have not developed CML like disease. Therefore, we hypothesize that (1) Increase of protein ISG15 modification in response to interferon is critical to the efficacy of interferon and (2) inhibiting UBP43 to increase protein ISG15 modification will significantly increase the efficacy of interferon in the treatment of CML. This grant funding is to demonstrate that protein ISG15 modification is crucial for interferon function in CML treatment and to analyze the effect of UBP43 on CML development.

Body:

Task 1. To demonstrate that protein ISGylation is crucial for interferon function in CML treatment:

- a. To generate UBE1L expressing K562 cells (Year 1 – 2).
- b. To study the effect of protein ISG15 modification on interferon responsiveness (Year 2 – 3).
- c. To analyze the correlation of protein ISGylation and interferon response in primary human CML samples (Year 1 – 3).

ISG15 is a small protein encoded by an interferon stimulated gene (ISG) (1-3). Its expression is highly induced upon interferon treatment. ISG15 is comprised of two domains, both of which have homology to ubiquitin (4). The N-terminal and C-terminal domains of ISG15 are 33% and 32% identical to ubiquitin, respectively. Upon interferon treatment, ISG15 can be detected in cells both in the free and conjugated form (5). In most cell types and tissues protein ISGylation is almost undetectable under normal conditions.

There is a series of distinct enzymes involved in the process of protein ubiquitination and deubiquitination, including ubiquitin activating enzyme (E1), ubiquitin

conjugating enzyme (E2), ubiquitin – protein ligase (E3), and the ubiquitin proteases (ubp) (6-8). In contrast, the enzymes involved in protein ISGylation have not been so well studied yet. A gene encoding a protein (UBE1L) homologous to the ubiquitin-activating enzyme E1 has been cloned during the analysis of chromosomal 3p21 deletions associated with small cell lung cancer (9). The chromosomal 3p21 deletion is also associated with non-small cell lung cancer and other solid tumors (10). Furthermore, immunohistochemical analysis has revealed that UBE1L is expressed in normal lung cells, but not in 14 human lung cancer cell lines (11). These studies indicate that UBE1L may play an important role in the prevention of cancer development. A recent study reports that the influenza B virus protein, NS1B, blocks protein ISGylation via its direct interaction with ISG15. Further analysis by the same group indicates that UBE1L is an E1 for protein ISG15 modification (12). We and another group have recently identified Ubc8 as ISG15 E2 (13,14). Most recently, we have identified that estrogen responsive finger protein (EFP) can function as an E3 enzyme in ISGylation (Zou et al. manuscript in preparation).

During the analysis of genes differentially expressed between wild type and leukemia fusion protein AML1-ETO knock-in mice, we have cloned a novel gene product and named it UBP43 (15,16). The predicted amino acid sequence indicates that UBP43 is a member of the UBP family of ubiquitin specific proteases. UBP43 contains the conserved domains, including the Cys and His domains, that are present in all UBP family members (6,7,17,18). In addition, it has little homology to other family members outside the two conserved regions. Our functional analysis of UBP43 demonstrated that it is an ISG15 specific protease (19). To understand the role of UBP43 and protein ISGylation, we generated UBP43 knockout mice. UBP43^{-/-} cells have higher interferon induced protein ISGylation than UBP43^{+/+} and ^{+/-} cells. Furthermore, UBP43^{-/-} cells are hypersensitive to interferon treatment. These findings lead to the hypothesis that inhibiting UBP43 enzyme activity during interferon cancer therapy may significantly enhance the efficacy of interferon. Furthermore, in contrast to the rapidly development of a myeloid proliferation disorder with BCR/ABL expressing wild type bone marrow cells, UBP43^{-/-} cells do not develop such disease, indicating that UBP43 plays a crucial role in the regulation of myeloid cell proliferation during leukemogenesis.

It has been reported that the expression of interferon stimulated genes were increased in both interferon sensitive and resistant CML patients, indicating the major defect of interferon resistance is not at the level of interferon signaling and is at the level of posttranslational modification (20). The K562 cell line is a hematological malignant BCR/ABL expressing cell line derived from a 53 year old female CML patient (21). Compared to many other cells, K562 cells are resistant to interferon induced suppression of cell proliferation (22). Since it has been reported that ISG15 activating enzyme UBE1L gene deletion is associated with small cell lung cancer development, we decided to study whether K562 cells lack UBE1L for ISG15 conjugation. After addition of a UBE1L protein expression construct into K562 cells by transient transfection, we can clearly detect ISGylated protein upon interferon treatment (23). This result indicates that lack of protein ISGylation may contribute to K562 cell resistance of interferon treatment. Therefore, we decided to establish K562 cell lines expressing UBE1L to study the role of protein ISGylation in interferon response of K562 cells and by directly investigating the

correlation of interferon response and protein ISGylation in primary CML patient hematopoietic cells as stated in task I.

In the second funding period, we have focused on the development of KT-1 cells that have lower levels of UBE1L expression and of KT-1 cells that have lower levels of UBP43 expression upon interferon treatment, due to the difficulties to generate inducible or stable UBE1L expression K562 cells. KT-1 is another BCR/ABL positive leukemia cell line. In contrast to K562 cells, KT-1 cells are sensitive to interferon treatment and have a strong increase of ISGylation upon interferon treatment. For generating KT-1 cells with lower UBE1L expression, we chose to use a RNA interference approach. Using the conventional rule for constructing an siRNA construct, we selected several regions of UBE1L cDNA and generated MSCV retroviral constructs that express short hairpin RNA of UBE1L under the control of U6 promoter (MSCV-U6-shUBE1L). As shown in figure 1A, when MSCV-U6-shUBE1L DNA was cotransfected with plasmids expressing HA tagged UBE1L and AML1, #1 shUBE1L clearly blocked the expression of UBE1L, but not the expression of the unrelated AML1. In contrast, #2 shUBE1L did not affect UBE1L expression. Therefore, we tested the function of this shUBE1L in human HeLa cells. HeLa cells were infected with MSCV-U6-shUBE1L retrovirus and their protein lysates were used in western blot to study the effect of shUBE1L on protein ISGylation upon IFN treatment. We detected a dramatic decrease of protein ISGylation in cells infected with shUBE1L retrovirus but not in cells infected with retrovirus vector (Fig. 1B). These results demonstrate that we can use RNA interference as an approach to lower the level of cellular protein ISGylation. Next, we infected KT-1 cells with MSCV-U6-shUBE1L retrovirus and established a pool of KT-1 cells stably expressing UBE1L siRNA after puromycin resistance selection. The levels of protein ISGylation in these cells were examined with protein lysates from cells with or without interferon treatment. As shown in figure 1C, we can detect a decrease of protein ISGylation in siRNA expressed cells. However, the level of decrease is not as strong as shown in HeLa cells. In the next funding period, we will make individual clones from the pool of UBE1L siRNA expressing cells and screen for KT-1 cell lines with the best inhibition of UBE1L expression and ISG15 conjugation. Then, these cells will be used to continue the proposed studies.

In the current funding period, we also generated KT-1 cells with even higher levels of protein ISGylation by expressing UBP43 siRNA in a pSuper-Retro retrovirus vector (pSR-shUBP43). As shown in figure 2A, UBP43 expression in KT-1 cells is highly induced by interferon treatment. However, no obvious signal of UBP43 expression is detected in the same northern blot when KT-1 cells have UBP43 siRNA via pSR-shUBP43 infection. Furthermore, UBP43 siRNA expressing KT-1 cells showed much higher levels of ISG15 conjugation and Stat1 phosphorylation (Fig. 2B), indicating the enhancement of JAK-STAT signal transduction upon interferon stimulation. As shown in figure 3, UBP43 siRNA expressing cells showed much slower initial proliferation rates. In the next funding period, these cells will also be used to study ISGylation and UBP43 in interferon responses.

Regarding the second portion of task I – to analyze the correlation of protein ISGylation and interferon response in primary human CML samples, as reported in the last progress report, we have established the conditions to perform the study. Due to the major effort spent in the above assays, we have not made much progress in this area. In

the next funding period, more effort will be spend on studying primary human patient CML samples.

Task 2. To analyze the effect of UBP43 on CML development.

- a. To study protein ISG15 modification and UBP43 expression in leukemic cells upon interferon stimulation (Year 1).
- b. To investigate the role of UBP43 in CML development (Year 1-3).
- c. To study interferon sensitivity of UBP43+ and UBP43- cells in the presence or in the absence of BCR/ABL expression (Year 2-3).

We have reported that UBP43 deficient cells are hypersensitive to interferon treatment. Our preliminary data have shown that UBP43 deficient hematopoietic cells are resistant to the development of BCR/ABL induced CML development in the retrovirus mediated bone marrow transplantation model. As shown in figure 4, we have completed the first part of the study and are ready to prepare a manuscript for publication. Mice transplanted with wild type bone marrow cells expressing BCR/ABL develop CML like disease within five weeks. When the mice become moribund, they generally have 10 to 100 times more total white blood cells in their peripheral blood and show splenomegaly and hepatomegaly. Furthermore, large amounts of infiltrated neutrophils at various developmental stages can be observed in Hematoxylin-Eosin (HE) stained liver and spleen sections (Fig. 4B). Interesting, not only are UBP43 deficient bone marrow cells more resistant to leukemia development (Fig. 4A), there are also much less neutrophil infiltrations in the liver and spleen when they eventually develop CML (Fig. 4B).

Interferon α is a useful drug to treat CML. Therefore, besides studying ISGylation in CML cells upon interferon stimulation as described in the previous year report, we also studied UBP43 expression in CML cells to investigate the role of UBP43 in interferon effects in CML treatment,. As shown in figure 5A, when mice developed the CML like disease, there is a 4-fold increase of type I interferon concentration in their sera. Furthermore, UBP43 expression is much higher in the spleen of CML mice compared to control spleen samples (Fig. 5B). These results, together with normal levels of interferon induced protein ISGylation in CML mice (presented in the previous report), indicate that leukemia per se does not block the expression of UBP43 and ISG15 nor the conjugation of ISG15 to target proteins upon interferon treatment.

To analyze the effect of UBP43 on CML development, we also studied the expression of BCR/ABL in the presence or the absence of UBP43 expression. As shown in figure 6, we have used three types of murine embryonic fibroblasts (UBP43+/+, +/-, and -/- MEFs) to generate BCR/ABL expressing cells via retrovirus mediated infection. Interferon triggers the expression of UBP43; STI571 blocks BCR/ABL kinase activity. We have examined the level of BCR/ABL protein by western blotting. We did not detect any significant difference of BCR/ABL protein in these cells (Fig. 6). However, further studies in hematopoietic BCR/ABL+ cells showed some interesting preliminary results (Fig. 7). B210 is a Ba/F3 cell line (murine B cell) that expresses BCR/ABL via pcDNA3-BCR/ABL plasmid transfection (24). K562 and KT-1 are two human cell lines derived from patients with t(9;22) and expressing BCR/ABL. B210 and KT-1 are

sensitive to interferon treatment for reducing cell proliferation; K562 are insensitive to interferon treatment. When KT-1 cells are treated with 1,000 units/ml interferon, we detected a clear decrease of BCR/ABL mRNA expression (Fig. 7A). Surprisingly, at protein level, we cannot detect any change of BCR/ABL in KT-1 cells upon interferon treatment (Fig. 7B). Furthermore, upon interferon treatment, KT-1 cells containing UBP43 siRNA did not show the decrease of BCR/ABL mRNA (Fig. 7A). Interestingly, with UBP43 siRNA, a clear decrease of BCR/ABL protein was observed in KT-1 and B210 (two interferon sensitive), but not in K562 (interferon insensitive) BCR/ABL+ cells (Fig. 7B). These data indicate a potential effect of UBP43 on BCR/ABL expression. However, it is still too preliminary to make a firm conclusion. In the next funding period, we will continue to analyze this effect to understand the role of UBP43 in the expression of BCR/ABL.

In this funding period, to investigate the role of type I interferon signaling in the resistance of UBP43 deficient cells to develop CML, we have generated UBP43 and type I interferon receptor R1 subunit (IFNAR1) double knockout mice from UBP43^{+/-} and IFNAR1^{+/-} mice. Furthermore, we have used bone marrow cells from these double knockout mice and relevant control mice to perform BCR/ABL retroviral infection-bone marrow transplantation assays. As expected, mice with BCR/ABL+ wild type bone marrow cells developed CML like disease within five weeks of bone marrow transplantation (Fig. 8). Unlike UBP43 single knockout mice, 100% of UBP43 and IFNAR1 double knockout mice developed CML-like disease by less than eight weeks. Although there is still a significant delay of disease development, we can conclude that type I interferon signaling plays a critical role in the resistance of CML development of UBP43 deficient hematopoietic cells, since disease development in UBP43 single knockout mice is delayed even longer (Fig. 4),

In the next funding period, we will continue to perform experiments as proposed in the original application and also plan to submit manuscripts with these reported data.

Key Research Accomplishments:

- Generated BCR/ABL+ KT-1 cells with lower levels of UBE1L expression.
- Generated and initiated the analysis of BCR/ABL+ KT-1 cells with lower levels of UBP43 expression.
- Finished pathology studies of CML mice with UBP43 deficient BCR/ABL+ bone marrow cells
- Examined the role of UBP43 in BCR/ABL expression.
- Analyzed the role of type I interferon signaling in the resistance of CML development of UBP43 deficient cells.

Reportable Outcomes:

We are in the process of preparing manuscripts with currently available data.

Conclusions:

During the past one year, we generated both UBE1L and UBP43 siRNA expressing KT-1 cells and demonstrated the much lower levels of UBE1L and UBP43 expression in these cells. Furthermore, we have started to analyze these cells for interferon effect on their proliferation, differentiation, and survival. Secondly, thorough analyses of CML mice derived from both wild type bone marrow cells and UBP43 deficient bone marrow cells demonstrate the resistance of CML development in the absence of UBP43. We also observed interesting effect of UBP43 on the expression of BCR/ABL protein in hematopoietic cells. Finally, we demonstrated that interferon plays a critical role in the resistance of CML development of UBP43 deficient cells.

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Appppendices:

Please see attached eight figures.

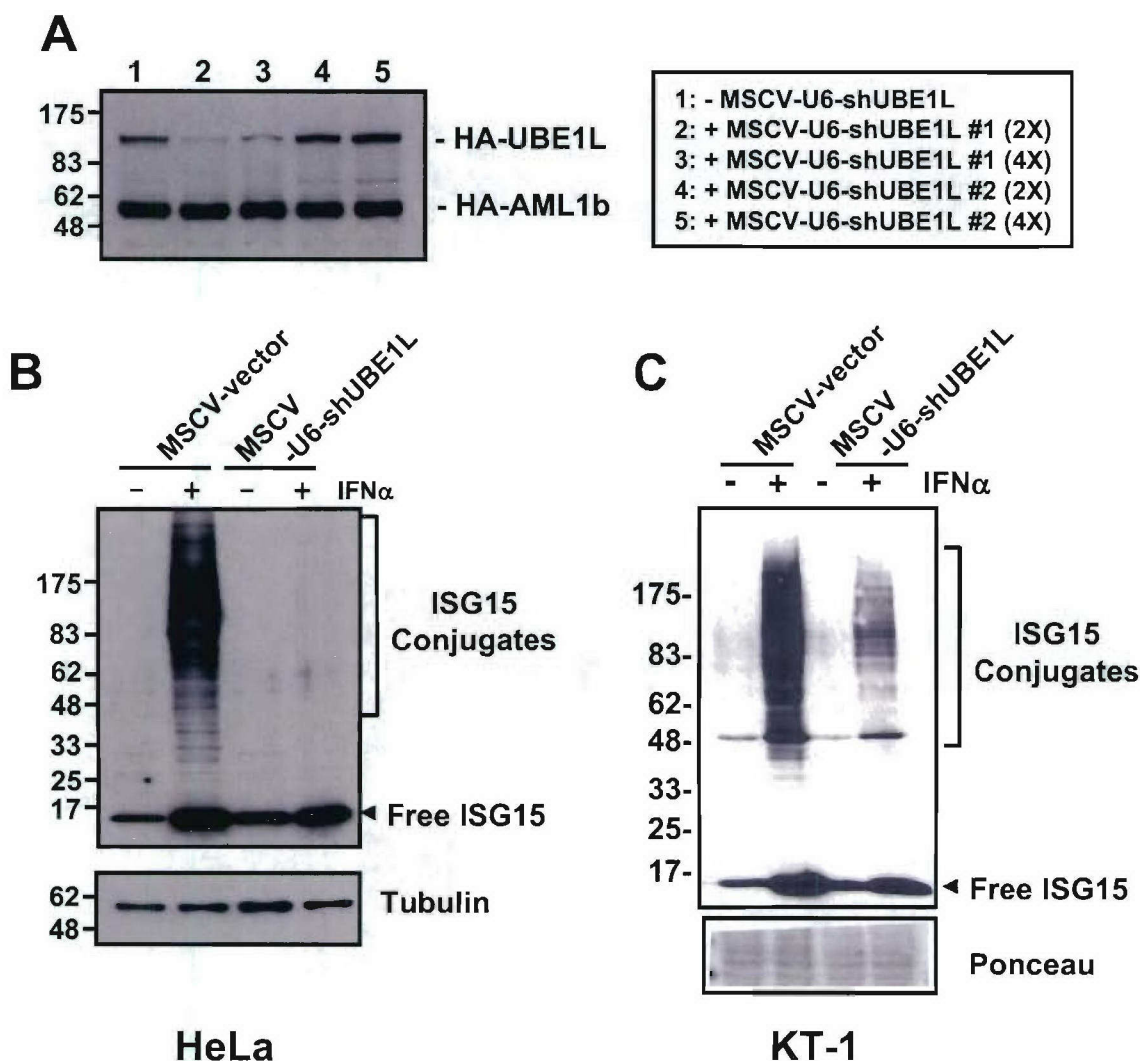


Figure 1. UBE1L activity can be inhibited by small interference RNA. 21 bp DNA sequences were selected from human UBE1L cDNA and were used to generate small hairpin RNA (shRNA) expression MSCV-U6-shUBE1L constructs as described in the text. (A) 293T cells were transfected with pcDNA-HA-UBE1L and pcDNA-HA-AML1 in the absence (Lane 1) or the presence of #1 shRNA (Lanes 2 & 3) or #2 (Lanes 4 & 5) UBE1L shRNA expression construct MSCV-U6-shUBE1L. 2x and 4x indicate the amount of MSCV-U6-shUBE1L DNA relative to pcDNA-HA-UBE1L DNA used in the transfection. HeLa cells (B) and KT-1 cells (C) were infected with MSCV control virus or MSCV-U6-shUBE1L#1 retrovirus. Infected cells were cultured in the presence or the absence of IFN for 24 hours. Antibodies against ISG15 were used in the western blots to detect the amount of ISGylated protein and free ISG15. Tubulin blot or ponceau stain is to show the relative amount of protein in each lane.

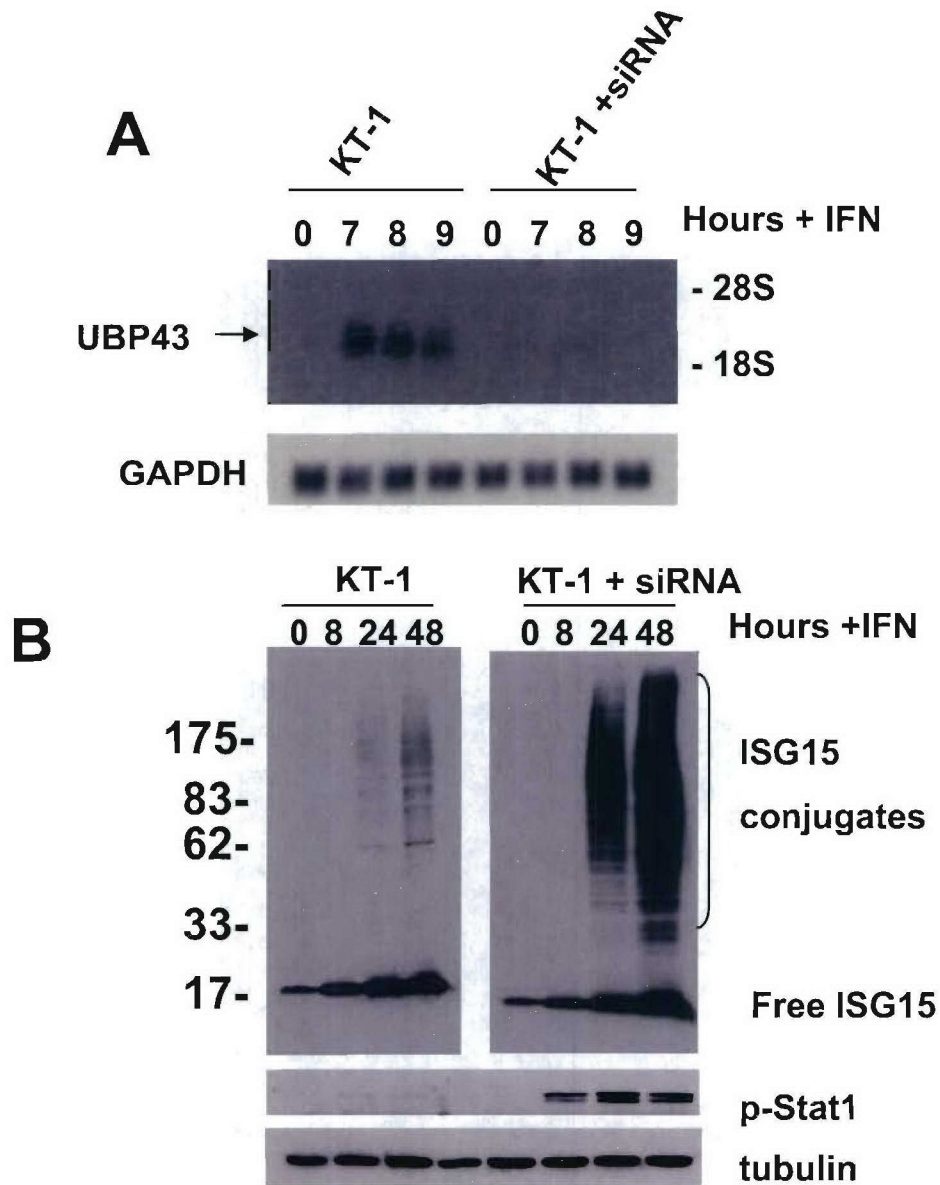


Figure 2. UBP43 activity can be inhibited by small interference RNA. 21 bp DNA sequences were selected from both murine and human UBP43 cDNA and were used to generate small hairpin RNA (shRNA) expression retroviral construct pSR-shUBP43. (A) KT-1 cells were infected with pSR-shUBP43. Stable pools were selected based on their drug resistance. RNA was prepared from non-infected and infected KT-1 cells after 1,000 units/ml of IFN α treatment for the time period as indicated in the figure. Northern blot is performed with radiolabeled UBP43 and GAPDH cDNAs. (B) protein lysates were prepared from parental and pSR-shUBP43 infected KT-1 cells after these cells were treated with 1,000 units/ml of interferon (IFN α) as indicated in the figure. Protein blot was hybridized sequentially with antibodies against ISG15, tyrosine 701 phosphorylated Stat1, and tubulin.

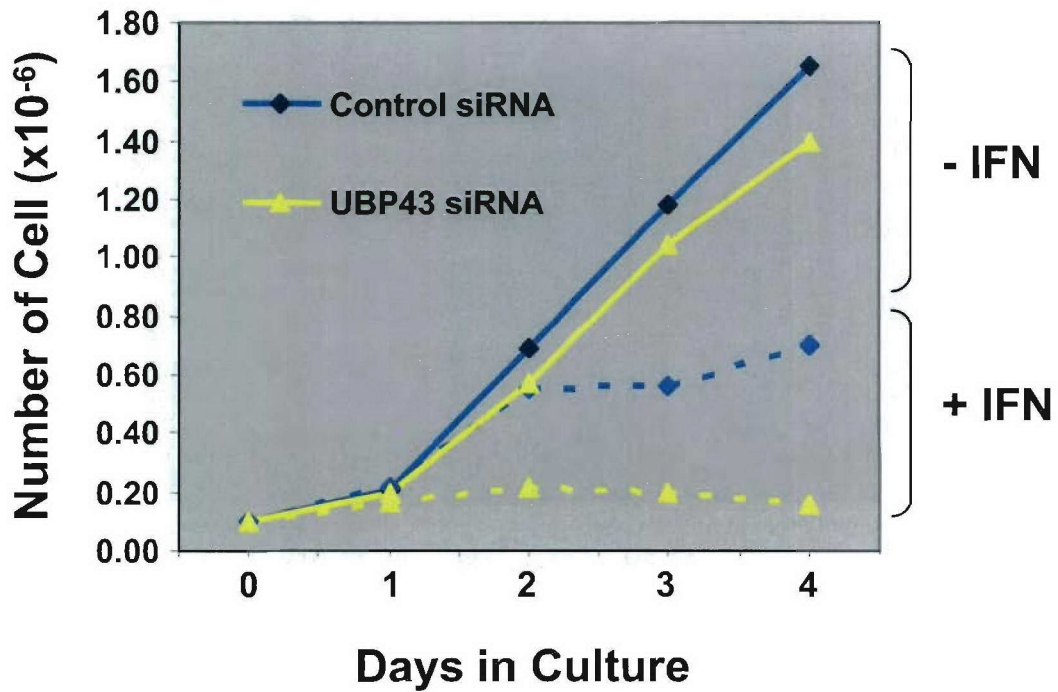


Figure 3. BCR/ABL positive KT-1 cells are hypersensitive to interferon treatment in the absence of UBP43. KT-1 cells are infected with retrovirus expressing either control siRNA or UBP43 specific siRNA. After drug selection, the pools of infected cells were splitted into 1×10^5 cells/ml and cultured in the presence or absence of 1,000 units/ml interferon (IFN α). Cells were counted every 24 hours.

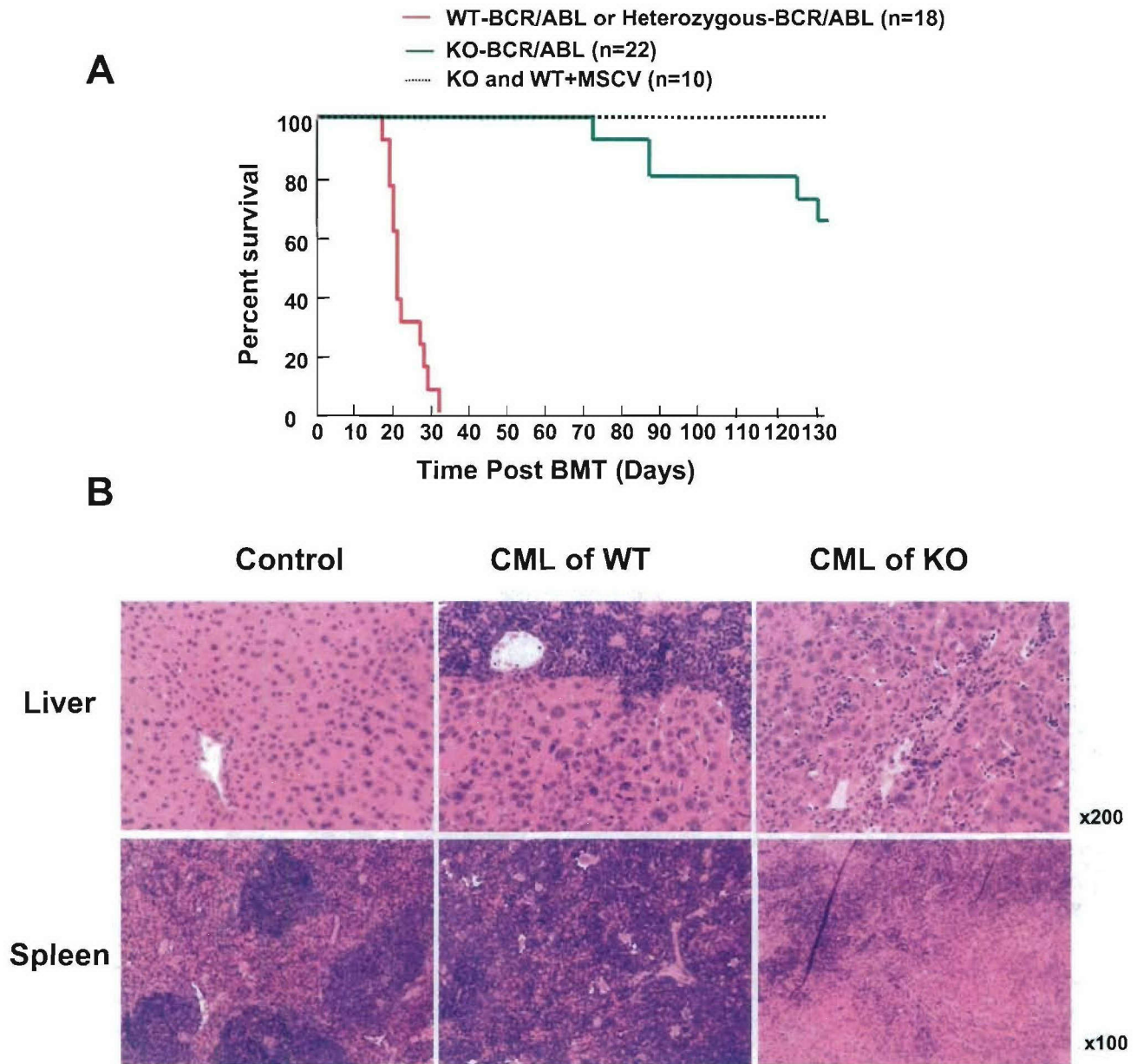


Figure 4. CML development with bone marrow cells from UBP43^{+/+} (WT), UBP43^{+/-} (heterozygous), and UBP43^{-/-} knockout (KO) mice. Bone marrow cells from WT, heterozygous, or KO mice were infected with MSCV vector or MSCV-BCR/ABL-IRES-EGFP retrovirus and transplanted into lethally irradiated recipient mice. (A) Survival curves of bone marrow transplanted (BMT) mice. There is a substantial delay of CML development when KO cells were used in BMT. (B) HE staining of liver and spleen sections prepared from healthy control and BCR/ABL CML mice derived from WT or KO bone marrow cells. A clear reduction of leukemia cell infiltration in liver and spleen is observed when UBP43^{-/-} bone marrow cells are used in the assay.

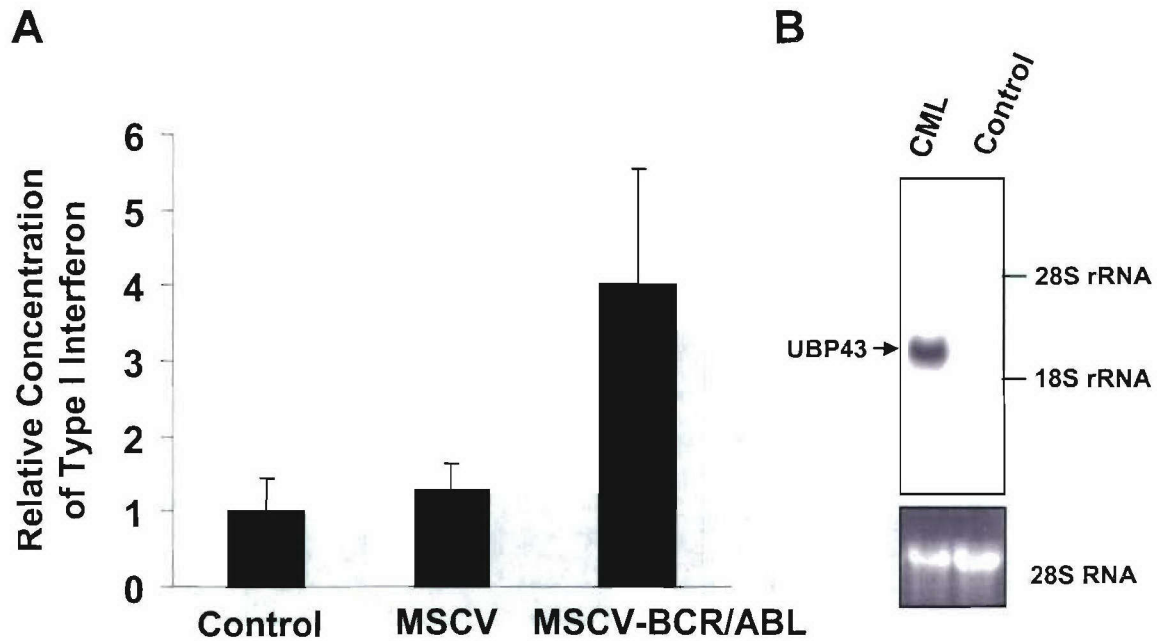


Figure 5. Increased levels of serum type I interferon and increased expression of UBP43 in CML mice. (A) The relative levels of type I interferon in the serum of wild type control mice, healthy mice transplanted with MSCV retrovirus vector, and CML mice induced by BCR-ABL expression via MSCV-BCR/ABL-IRES-EGFP. (B) Northern blot hybridization to measure the expression of UBP43 in the spleen of CML and healthy control mice.

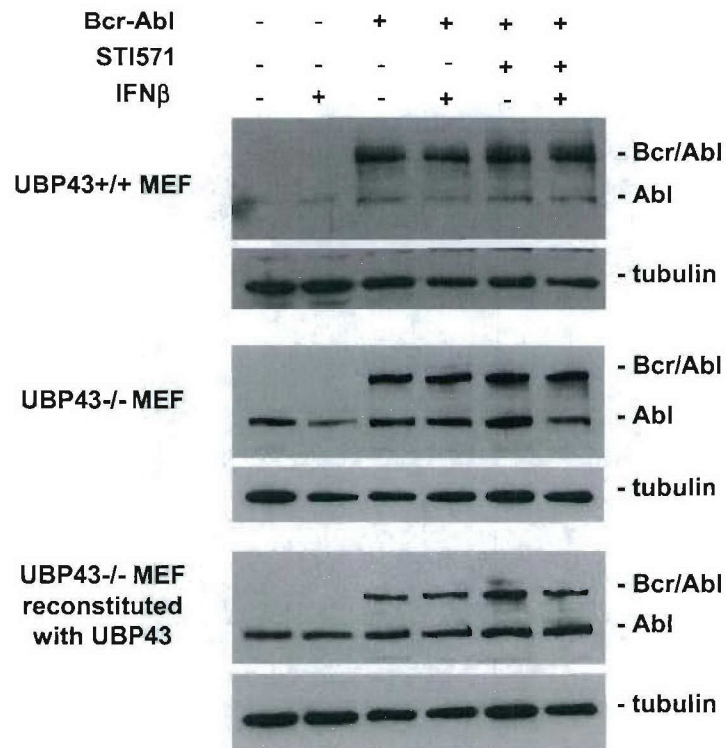


Figure 6. UB43 does not show any effect on the levels of BCR/ABL expression in mouse embryonic fibroblasts (MEF). MEFs of UB43^{+/+}, UB43^{-/-}, and UB43^{-/-} reconstituted with UB43 were infected with BCR/ABL expressing retrovirus MSCV-BCR/ABL-pGKneo and cultured in the presence or absence of 1 μ g/ml STI571, 500 units/ml interferon (IFN β) for 48 hours. Cell lysates were used in western blotting to hybridize sequentially with antibodies against Abl or tumulin.

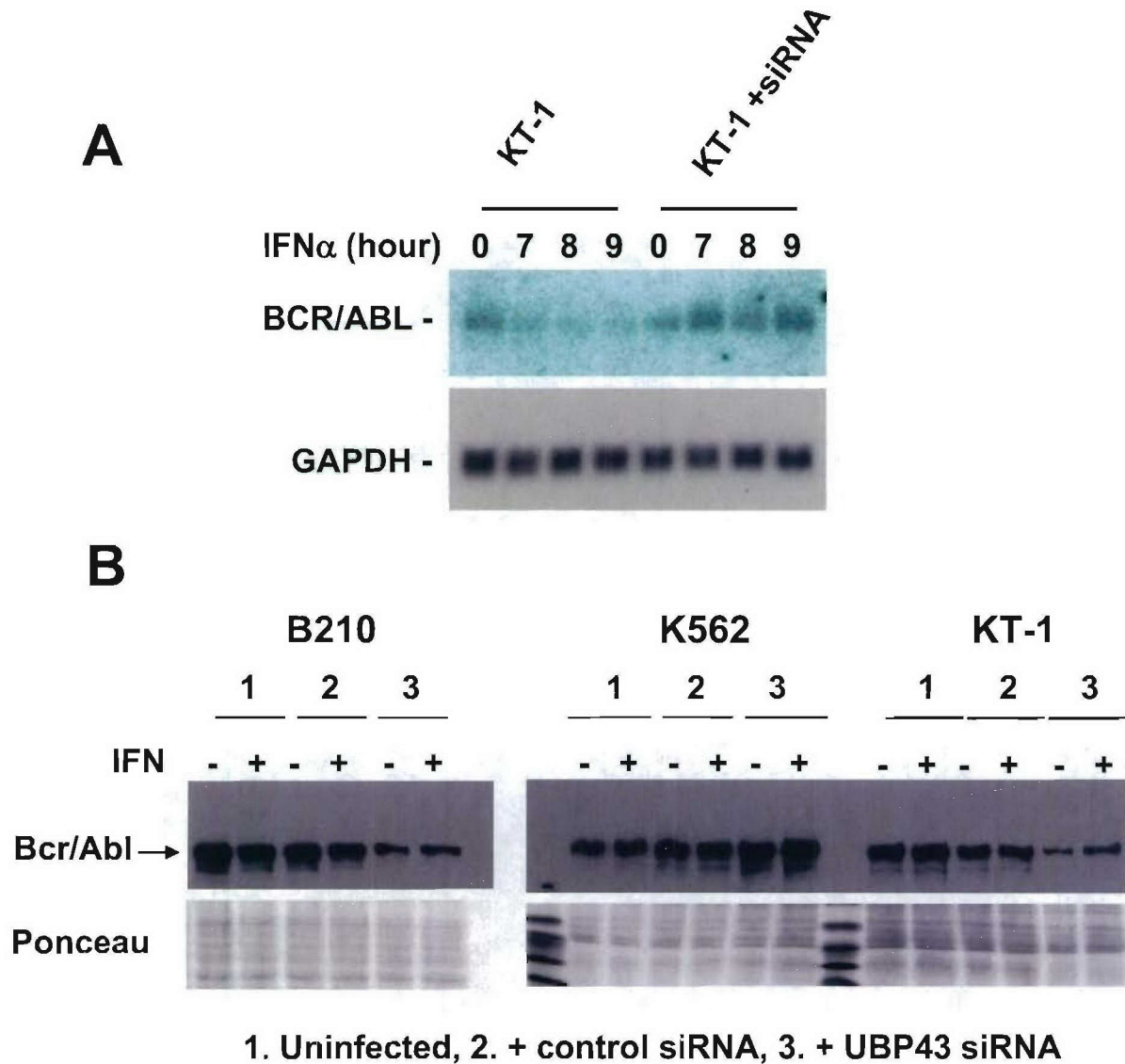


Figure 7. Examination of effect of UBP43 on BCR/ABL expression. (A) Northern blotting was performed with RNA prepared from KT-1 cells and KT-1 cells expressing UBP43 siRNA upon interferon stimulation. (B) Western blotting was conducted to study BCR/ABL protein expression in three BCR/ABL expressing cell lines without or with UBP43 siRNA expression. In B210 cells, BCR/ABL is exogenously expressed with an artificial promoter construct. K562 and KT-1 cell lines are developed from BCR/ABL positive leukemia cells of patients. B210 and KT-1 are sensitive to interferon treatment; K562 cells are insensitive to interferon treatment. Ponceau staining is used to indicate the relative loading of protein samples.

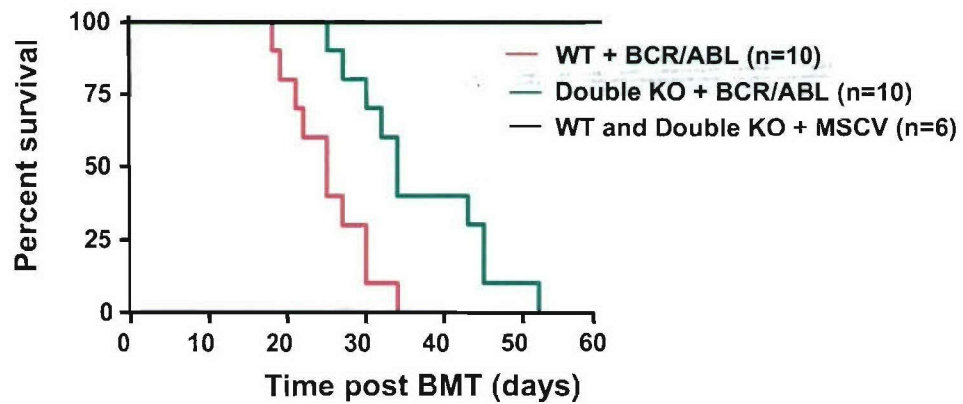


Figure 8. Type I interferon signaling plays a critical role in the resistance of CML development of UBP43 knockout cells. Bone marrow cells from wild type (WT), UBP43 and type I interferon receptor R1 (IFNAR1) double knockout (KO) mice were infected with either retrovirus vector MSCV or retrovirus MSCV-BCR/ABL-IRES-EGFP and were transplanted into lethally irradiated mice. CML development and survival of these mice were closely monitored.