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The ups and downs of p53 regulation in hematopoietic stem cells

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Hematopoietic stem cells provide an indispensible source for replenishing the blood with all its constituents throughout the organism's lifetime. Mice with a compromised hematopoietic stem cell compartment cannot survive. *p53*, a major tumor suppressor gene, has been implicated in regulation of hematopoiesis. In particular, p53 plays a role in homeostasis by regulating HSC quiescence and self renewal. We recently utilized a hypomorphic $p53^{515C}$ allele in conjunction with Mdm2, a negative regulator of p53, to gain insights into the role of p53 in hematopoietic regulation. Our analyses revealed that $p53^{515C/515C}Mdm2^{-/-}$ double mutant mice die soon after birth due to hematopoietic failure. Further mechanistic studies revealed that in the absence of Mdm2, ROS-induced postnatal p53 activity depletes hematopoietic stem cells, progenitors and differentiated cells.

Key words: HSC, reactive oxygen species, ROS, p53, Mdm2

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The p53 Tumor Suppressor in Hematopoiesis

The p53 tumor suppressor and transcription factor is activated in response to DNA damage signals including ionizing radiation (IR), hypoxia, nucleotide tri-phosphate (NTP) depletion and reactive oxygen species (ROS).[1] Active p53 impacts multiple pathways as it transactivates genes involved in apoptosis, cell cycle arrest, senescence, metabolism, fertility and differentiation. Not surprisingly then, p53 levels are constantly regulated in a cell. Mdm2 and Mdm4 play major roles in this regulation as genetic ablation of either gene in mice results in embryonic lethal phenotypes that are rescued by deletion of both *p53* alleles.[2-4]

Hematopoietic defects are observed in mice with *Mdm2* hypomorphic and null alleles that express approximately 30% the total levels of *Mdm2*.[5] $Mdm2^{+/-}$ $Mdm4^{+/-}$ mice also succumb to p53-dependent hematopoietic defects and die shortly after birth.[6] Additionally, while $Mdm2^{+/-}$ and $Mdm4^{+/-}$ mice are normal and viable, they are highly sensitive to sublethal irradiation and succumb to bone marrow ablation within days after radiation.[6] Mice with enhanced p53 activity due to a mutant p53 allele ($p53^{m}$) display deficiency in hematopoietic engraftment.[7] On the other hand, *p53*-null mice and mice treated with pifithrin-α, a chemical inhibitor of p53 activity, do not succumb to radiation induced hematopoietic syndrome.[8] Similarly, mice with homozygous deletion of *Puma* or *Bax*, two major p53 target genes that induce an apoptotic response, are overtly radio-resistant.[9,10] These genetically engineered mice do not undergo typical degeneration of the hematopoietic organs after radiation exposure. Thus, these data support an essential role of p53 in development and maintenance of the hematopoietic system.

Dissecting the Hematopoietic Hierarchy

The hematopoietic system is one of the most well-studied systems in normal physiology.[11] Hematopoietic stem cells (HSCs) are at the apex of the hematopoietic system. HSCs give rise to short-term progenitors that produce more committed progenitors, namely common myeloid progenitors (CMP) and common lymphoid progenitors (CLP).[12,13] CMP and CLP produce the committed

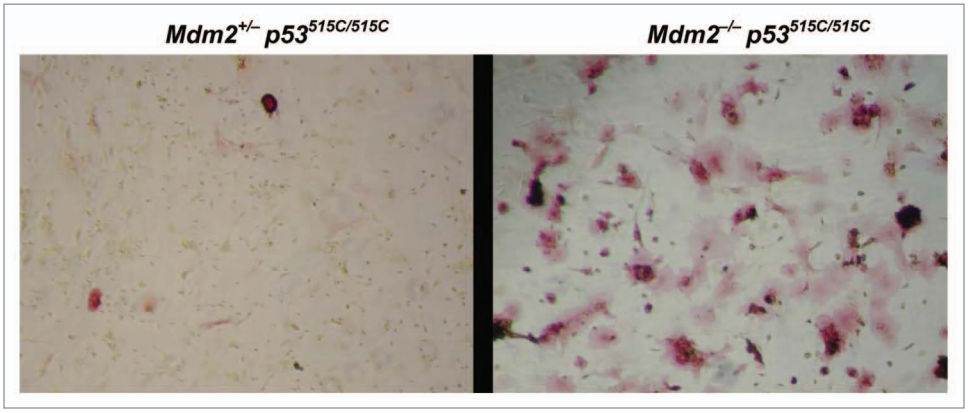

Figure 1. Increased osteoblastic differentiation of P6 bone marrows of *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ mice in vitro. P6 whole bone marrows were allowed to grow for MSC colony formation. *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ (right) cells spontaneously differentiated into osteoblastic lineage as seen by red alkaline phosphatase activity while *Mdm2*$^{+/-}$ *p53*$^{515C/515C}$ (left) retained undifferentiated state.

precursors which subsequently differentiate into mature hematopoietic cells that are relatively short-lived. HSCs repeat this cycle throughout their lifetime in order to replenish the blood and bone marrow with appropriate cell types, while still maintaining their ability to self-renew.[11,14] HSCs are routinely identified using Lin$^-$cKit$^+$Sca1$^+$ (LKS) markers. These HSCs when transplanted in lethally irradiated mice reconstitute the bone marrow and allow survival of the organism.

The developmental origins and the processes of hematopoiesis are generally conserved among vertebrates.[11] Most of these processes have been delineated from studies in mice. Hematopoiesis progresses at different anatomical sites during embryogenesis and adulthood.[14] Hematopoiesis initially starts in the yolk sac at around embryonic day (E) 8 in mice, shifts to the fetal liver at E12.5, and finally the bone marrow at E16.5.[15,16] The bone marrow remains the major hematopoietic organ after birth and in adult mice. The molecular and signaling pathways that regulate HSC function and activities remain unidentified.

p53 in Stem Cell Regulation

Recent studies have emphasized the role of p53 in regulation of stem cells. Expression of Nanog, a gene regulating self-renewal of embryonic stem (ES) cells, is regulated by p53.[17] Also, loss of *p53* diminishes spontaneous apoptosis and differentiation of ES cells.[18] More recently, several laboratories have shown that loss of *p53* improves the generation of induced pluripotent stem cells (iPSC) from adult cells.[19-23]

In HSCs, p53 is preferentially expressed in LKS cells where it negatively regulates self-renewal and maintains quiescence.[24] Mice deficient in *p53* show enhanced HSC self-renewal and have an increased HSC pool size.[24] Recent studies have provided further evidence of p53 function in HSC regulation. Loss of MEF, a positive regulator of the p53 inhibitor Mdm2 in mice results in increased p53 activity and enhanced stem cell quiescence.[25] Similarly, overexpression of miR-33, a microRNA which negatively regulates p53, enhances the transplantation efficiency of wild-type HSCs.[26,27] Conversely, mice harboring an extra genomic copy of p53 (super-p53) have lower levels of miR-33 and HSCs compared with wild-type mice.[26,27] Lastly, p53 also negatively regulates neural stem cell proliferation and self renewal.[28] Thus, p53 is an important regulator of stem cell behavior.

Mdm2$^{-/-}$ *p53*$^{515C/515C}$ as a Model for p53's Role in Hematopoiesis

Numerous *p53* mutant mice have been generated.[29] One in particular contains a *p53* allele (*p53*^{515C}) encoding the p53R172P protein that retains the ability to induce cell cycle arrest and senescence, but not apoptosis.[30,31] To delineate the role of p53 cell cycle arrest and senescence activities in the absence of *Mdm2*, *p53*$^{515C/C}$ and *Mdm2*$^{+/-}$ mice were intercrossed.[32,33] Interestingly, *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice were born at normal Mendelian ratios but died before weaning. Histological examination of hematopoietic organs revealed a normal cellularity of fetal liver at E14.5 but a progressively acellular bone marrow after birth. Additionally, immunohistochemical analyses revealed that low p53R172P levels were present in fetal livers of *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ and the control *Mdm2*$^{+/-}$*p53*$^{515C/515C}$ littermates.[33] However, soon after birth, p53R172P levels became elevated in bone marrows of *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ mice, and this increase was coupled with severe depletion of hematopoietic cells. Since p53 is a stress-response gene, these data suggest that a stress signal instigated the increase in p53R172P levels and activity.

ROS as a p53 Activating Signal

Oxygen tension is a major difference between pre- and post-natal mice and we surmised ROS may be different pre- and post-birth. ROS damages DNA and hence p53 is activated in response to this stress.[34] To characterize whether ROS was the stress signal for p53R172P stabilization, we first analyzed ROS levels in embryonic E14.5 fetal livers and postnatal day (P) 6 bone marrows. As expected, P6 bone marrows from *Mdm2*$^{+/-}$*p53*$^{515C/515C}$ mice had a 3.8-fold higher ROS level compared with the corresponding fetal liver samples.[33] Surprisingly, the postnatal *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ bone marrows had 3.3-fold higher ROS levels than the

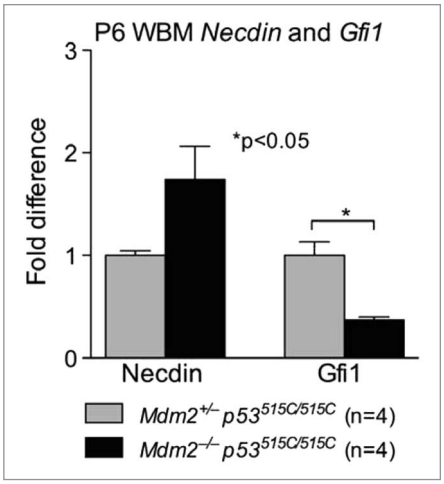

Figure 2. *Necdin* and *Gfi1* RNA levels in *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ mice compared with control *Mdm2*$^{+/-}$ *p53*$^{515C/515C}$ littermates. Necdin levels were not different among genotypes, but Gfi1 levels were significantly lower in moribund mice.

Mdm2$^{+/-}$*p53*$^{515C/515C}$ controls. These data suggest that adequate Mdm2 protein levels in *Mdm2*$^{+/+}$*p53*$^{515C/515C}$ and *Mdm2*$^{+/-}$ *p53*$^{515C/515C}$ dampen p53 activity. However, in the absence of Mdm2-mediated inhibition, ROS-induced p53R172P activity depleted all HSCs and progenitors cells in *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ bone marrows.[33] In agreement with these data, we could revive the hematopoietic potential of *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ hematopoietic cells by culturing them at low oxygen levels.[33]

Moreover, as p53R172P is defective for inducing apoptosis,[30] these data suggest that other modes of cell loss are involved in ablation of hematopoiesis in *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ mice. A significant increase in senescence markers *p21*, *Dcr2* and *p15* also implicate senescence as a possible pathway in abrogation of hematopoiesis.[33] These data are corroborated by higher SA-β*gal* activity in freshly isolated postnatal bone marrows from *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice as compared with *Mdm2*$^{+/-}$*p53*$^{515C/515C}$. *p21* loss partially rescued the postnatal lethality of *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice. Also, p53 activated ROS inducing genes, dubbed p53-induced genes (PIG), which are involved in inducing cell death.[33,35] Finally, we could rescue the bone marrow cellularity of postnatal *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ pups by injection of N-Acetyl-Cysteine (NAC), an antioxidant that antagonizes ROS. These data confirmed that ROS contributes to the hematopoietic defect in *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice through activation of p53R172P. Since *Mdm2*$^{+/-}$*Mdm4*$^{+/-}$ mice with wild-type *p53* have a similar hematopoietic phenotype,[6] these data may not necessarily be limited to the p53R172P protein but are also likely a characteristic of wild-type p53 activity.

Other Models of HSC Defects Due to ROS

Elevated ROS in HSCs is correlated with defects in other pathways. For instance, HSCs of *Atm*$^{-/-}$ mice accumulate ROS which limits the self-renewal capacity of HSCs in serial transplantation experiments.[36] Inappropriate activation of mTOR due to targeted mutation of its negative regulator *Tsc1* stimulates mitochondrial ROS biogenesis, concomitant with increased cycling of HSCs and ultimately loss of HSC "stemness."[37,38] Also, *Bmi1* deletion abrogates mitochondrial function and instigates higher ROS levels, thus damaging HSC function.[24] Deletion of *Chk2*, a DNA damage response gene and activator of p53, alleviates the hematopoietic defect of *Bmi1*-null mice.[24] Based on our model, it is plausible that the *Bmi1* effect is mediated through Chk2 activation of p53. This is further supported by the partial rescue of *Bmi1*-loss on hematopoiesis by deletion of *p53*.[39] Our model suggests that p53 activation eventually leads to increased transcription of a set of ROS-inducing genes which results in depletion of HSCs and their progenitors, subsequently causing cell death.

Possible Mechanisms for ROS-Dependent p53 Activation in HSCs

Evidently, elevated ROS levels negatively influence HSC and progenitor activities. But how exactly is this achieved? Recent studies in Drosophila demonstrate that elevated ROS levels in myeloid progenitors induce their differentiation via downregulation of Polycomb complexes and upregulation of *Jnk* and *Foxo* signaling pathways.[40] Since p53 is clearly implicated in differentiation,[41] this remains a plausible effect of p53R172P-dependent ROS production on HSCs, leading to

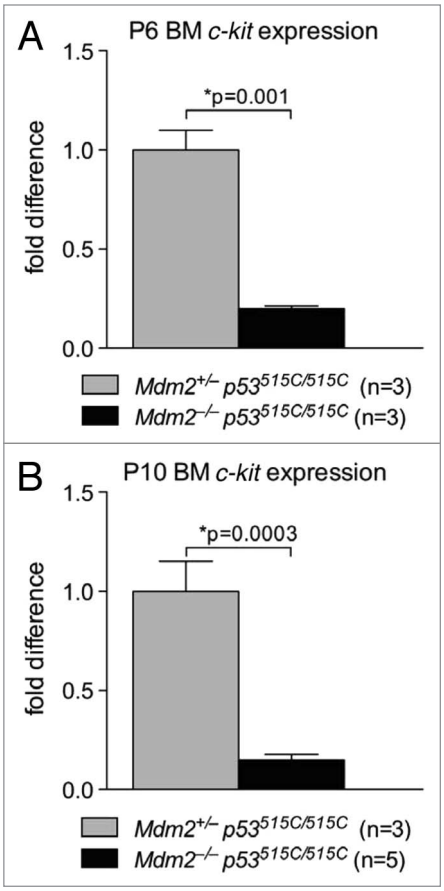

Figure 3. *c-kit* mRNA levels are significantly lower at P6 and P10 in *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ bone marrows. c-kit mRNA expression measured with RT-qPCR at P6 (A) and P10 (B) of whole bone marrow (BM) cells from *Mdm2*$^{+/-}$ *p53*$^{515C/515C}$ and *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ mice. Error bars represent standard error of the mean.

their eventual disappearance in *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ mice. Specifically, rapid differentiation of HSCs into progenitors or differentiation of progenitors into more differentiated cells would result in short-lived cells that die off within days. Testing such a hypothesis would require lineage tracing of *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ HSCs after sorting. However, the low number of HSCs in our mouse model remains an obstacle to addressing this question.

Similarly, p53 is also implicated in mesenchymal lineage differentiation.[42,43] To examine possible effects on mesenchymal differentiation, we collected P6 whole bone marrows of *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ mice and control littermates, and plated them at low cell density using mesenchymal differentiation specific media. Typically, cells are plated for a few days until fibroblast-like

colonies form which are then induced to differentiate by adding lineage specific factors to the media. Surprisingly, cultures of *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ bone marrows differentiated into osteoblastic lineage as seen by increased alkaline phosphatase staining without addition of lineage specific factors (**Fig. 1**). These data indicated that activation of p53R172P in the absence of *Mdm2* could also alter differentiation capacity leading to defective hematopoiesis.

Alternatively, as in the *Atm*$^{-/-}$ mouse model,[36] elevated ROS could have abrogated the self-renewal capacity of HSCs in *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice. ROS-dependent depletion of bone marrow cellularity could have exhausted the HSC pool and impaired their self-renewal capacity while trying to regenerate bone marrows. This is supported by the failure of HSCs from P6 bone marrows of *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ to rescue lethally irradiated recipients.[33] However, more studies are required to address whether the failure of *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ HSCs to reconstitute hematopoiesis is due to intrinsic or extrinsic defects. One approach could be to transplant *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ HSCs from E14.5 fetal livers (when HSCs numbers and ROS levels are normal) into lethally irradiated mice. Also, to test whether the *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ niche is intact, wild-type HSCs can be transplanted into *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ neonates. These experiments would importantly differentiate between defects that could be intrinsic to HSCs, their niche or both. In the light of the role of p53 in altering mesenchymal lineages, it would not be surprising to find that the niche is also defective. In summary, a p53-dependent increase of ROS levels in HSCs obstructs their normal function, although exactly how this happens is still not perfectly clear.

Quiescence Markers in *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ Bone Marrows

Slow cycling hematopoietic stem cells are associated with hypoxic regions of capillaries suggesting that low ROS maintains quiescence and self-renewal.[44] *Necdin* and *Gfi-1* are newly identified targets of p53 in HSCs that induce HSC quiescence.[24] In *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice, the frequency of LKS is quite low impeding the measurement of RNA levels of these target genes by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). We therefore isolated RNA from whole bone marrows of neonatal mice. We examined *Necdin* and *Gfi-1* levels in whole tissues and did not see any differences in *Necdin* levels at P6, but *Gfi1* levels were lower in *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ cells as compared with control *Mdm2*$^{+/-}$*p53*$^{515C/515C}$ cells (**Fig. 2**). The significance of lower *Gfi1* in *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice warrants further investigations.

Table 1. List of primers used to span the c-kit promoter for p53 binding

Primer	Forward primer	Reverse primer
ckitRE 1	CTC CAG GTG CGC TAT GCA	TGG GTG CTT TGC CTG TTT CT
ckitRE 2	TGT AGC GCC AGC ACT TGT G	AGC TGA GGA TGG CTT TGA ACT C
ckitRE 3	CCA ACA GAG CAA CAC AAA GCA	GAA TAG GTT TCC CCC TCC ATC T
ckitRE 4	GCG CAG CGT TCA ACC TGT A	CCT GAG ACA CCC ACC TCA CA
ckitRE 5	CAG GGC TCC CAT CTC AGA TC	AGC GAG GCA CTG TTA GTA GAT GTG
ckitRE 8	TGG AGA AAC TGA GCA TGA AAA ATT	GCA CCC TGA CCT CAG AAA AG
ckitRE 9	CCG GTG GTT GTC CTT TAT TGT C	GCC ACG AGC GCA TTA GGT A
Puma	GGA CGG TCG CCT TGC A	CAC CTT AGT CCC AGT GAT GAA A
AchR	CC TCC CCC AAC TCC ACT TTT	GGA GGT TGG AGG GAG AAG GA

Puma and Acetylcholine Receptor (AchR) primers sets were used as positive and negative controls respectively.

p16: At the Crossroads of ROS, p53 and HSCs

p16 overexpression in HSCs inhibits their proliferation purportedly via ROS regulatory pathways that are still poorly understood.[36,39,45] Our study further supports the possibility that ROS induces p16 expression and exacerbates the phenotype in *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice. p16 is also implicated in senescence.[46] Senescent cells generate ROS which could create a positive feedback loop further stabilizing p53R172P and abrogating hematopoiesis.[33,46] Since *p16* deletion only partially rescues hematopoiesis and the survival of *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice, further studies are needed to address the importance of other players affecting HSC function. In a recent study, *Cited2* deletion increased p53 levels dampening the HSC numbers which could be rescued by deletion of both *p16* and *p19*^{ARF}.[47] It would be interesting to measure ROS levels in *Cited2*$^{-/-}$ HSCs and bone marrows to delineate the physiological mechanisms for HSC loss.

c-kit Downregulation in *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ Bone Marrows

c-kit is a major tyrosine kinase receptor expressed in hematopoietic stem cells and myeloid progenitors.[48] c-kit activation induces self-renewal, differentiation and expansion of hematopoietic stem cells. Loss of c-kit abrogates hematopoiesis, and is detrimental to development; its overexpression is oncogenic.[49,50] Specifically, *c-kit* mutant mice have severe hematopoietic defects and die shortly after birth, reminiscent of *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ mice.[51,52] Hence, we measured *c-kit* RNA levels using RT-PCR in P6 and P10 bone marrows of *Mdm2*$^{-/-}$*p53*$^{515C/515C}$ and found a 5-fold ($p = 0.0001$) and 7-fold ($p = 0.0003$) drop in c-kit RNA levels compared with control *Mdm2*$^{+/-}$*p53*$^{515C/515C}$ littermates (**Fig. 3A and B**).

We therefore hypothesized that p53R172P binding to a *c-kit* response element could be inhibiting its transcription. We screened the promoter region upstream of *c-kit* transcriptional start site (~5,000 bases) with 7 different ChIP primer sets (**Table 1**). However, we could not detect any significant binding of p53 on the c-kit promoter (data not shown). Thus the mechanism by which c-kit is regulated is unknown. Regardless, we believe that loss of c-kit expression is a significant modifier of the *Mdm2*$^{-/-}$ *p53*$^{515C/515C}$ hematopoietic phenotype given the consistent and severe decrease in its levels.

Implications to Leukemia Treatment

Aberrant regulation/expression of p53 is common in hematologic malignancies. *p53* deletions and mutations have been reported in acute leukemias and in chronic myelogenous leukemia in blast crisis.[53] Self-renewing normal HSCs and leukemia stem cells (LSCs) share multiple features.[54,55] For instance, both cell types express CD34, lose expression of CD38, are dormant early and can give rise to progeny that are more differentiated.[54] Other studies have also indicated that LSCs could very well originate from normal HSCs.[56] These data, however, do not rule out the possibility of transformation of a committed progenitor or differentiated cell in the blood lineage into a LSC. Leukemiagenesis proceeds via uncontrolled proliferation and differentiation, two pathways which are regulated by p53. Accordingly, it is plausible that pathways that sensitize normal HSCs could have a similar effect on LSCs. Our results, that Mdm2 acts as a critical regulator of p53 in the hematopoietic system, imply that careful monitoring of hematopoiesis should occur in leukemia patients when treating with drugs that target p53-Mdm2 association or re-activate wild-type p53.

Acknowledgments

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