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ROLES FOR THE DNA DAMAGE CHECKPOINT PROTEIN HUS1 IN BREAST CANCER

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<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> Cancer is aberrant, uncontrolled cellular proliferation arising from an accumulation of mutations in growth regulatory genes. Two mammalian DNA damage checkpoint pathways, the Atm and Atr pathways, act to suppress tumor formation by preventing mutation accumulation and inducing senescence in response to oncogenic stimuli. Roles for the Atr pathway in tumor suppression are less understood, as deletion of any member of this pathway, including Hus1, results in embryonic lethality. To understand roles for Hus1 in breast cancer suppression, we developed mouse models featuring partial Hus1 impairment and are testing how Hus1 dysfunction affects cellular responses to activated oncogenes. Proliferation, immortalization, focus formation, soft agar, and transplantation assays suggest that cultured cells with reduced Hus1 levels are less able to be transformed. To elucidate roles for Hus1 as a tumor suppressor <i>in vivo</i> , mice expressing reduced levels of Hus1 were crossed to mice overexpressing ErbB2 in the mammary gland to generate a cohort of mice. Our preliminary results indicate that reduced Hus1 levels may decrease the capacity of cells to undergo transformation, suggesting that Hus1, or the Atr pathway, may be a possible target for breast cancer treatment.						
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## Introduction:

Cancer arises from aberrant, uncontrolled cellular proliferation due to an accumulation of mutations in growth regulatory genes (1). An increase in the rate of mutation accumulation, termed genomic instability, can drive tumor formation and is particularly important in the genesis of breast cancer, as defects in several genes that normally function to preserve genomic integrity cause increased breast cancer risk (2). DNA damage checkpoints are a key genome maintenance mechanism and suppress tumor formation by preventing mutation accumulation and also by triggering senescence in response to oncogenic stimuli. The two primary mammalian checkpoint pathways center on the large protein kinases, Atm and Atr (3). Mutations in the better-characterized Atm pathway are well known to confer an increased risk of many cancers, including breast cancer (2, 4). The Atr pathway, by contrast, is essential for organismal viability and thus is far less well understood, but nevertheless has been hypothesized to function in tumor suppression (5-7). Hus1, an essential member of the Atr pathway, is a component of the Rad9-Rad1-Hus1 (9-1-1) heterotrimeric, PCNA-like sliding clamp, which is recruited to sites of DNA damage and is necessary for optimal phosphorylation of the Atr target and checkpoint effector Chk1 (8). In order to study the effects of Hus1 dysfunction while bypassing the embryonic lethality associated with complete Hus1 inactivation, our lab developed an allelic series which expresses incrementally reduced levels of Hus1 (9). Specifically, Hus1<sup>+</sup> mice express wild-type levels of Hus1 from one allele, and serve as control mice in our assays. Hus1<sup>Neo/ $\Delta$ 1</sup> mice express the lowest level of Hus1, while Hus1<sup>Neo/Neo</sup> mice have a less severe Hus1 impairment. The objective of this project is to use the Hus1 allelic series to determine how partial Hus1 impairment impacts neoplastic transformation in cultured cells and mammary tumorigenesis in mice. This study will provide critical new insights into how checkpoint dysfunction, and specifically the Atr pathway, influences breast carcinogenesis and will establish new breast cancer mouse models that may be of great use for evaluating therapeutics.

## Body:

### Task 1. Determine the effects of reduced Hus1 levels on cell transformation by activated oncogenes

- a. Cell proliferation assay: In order to determine the effect of reduced levels of Hus1 on cell transformation, primary cells expressing incrementally reduced levels of Hus1 were infected with activated oncogenes, and short term proliferation was assessed by cell counting. An increase in the number of cells, which results from proliferation, is an indication of transformation. Primary mouse embryonic fibroblasts (MEFs) from the Hus1 allelic series are being maintained in normal and low oxygen conditions. These cells were infected with viruses expressing GFP, activated H-Ras, or both activated H-Ras and c-Myc. Population doubling assays were carried out. In this assay, primary MEFs expressing wild-type or reduced levels of Hus1 were plated in a 6-well dish and infected with either GFP or H-Ras and c-Myc. Cell proliferation was measured by counting surviving cells every two days for 12 days. In this short-term proliferation assay, Hus1<sup>+/+</sup> cells infected with Ras and Myc showed enhanced proliferation when compared to Hus1<sup>+/+</sup> cells infected with GFP. On the other

- hand, Hus1<sup>Neo/Neo</sup> cells infected with Ras and Myc failed to proliferate beyond Hus1<sup>Neo/Neo</sup> cells infected with GFP (Fig. 1). These preliminary results suggest that reduced Hus1 results in resistance to oncogene induced proliferation. This assay will be repeated using primary MEFs that express the lowest level of Hus1, Hus1<sup>Neo/ $\Delta$ 1</sup> MEFs.
- b. **Immortalization assay:** In order to determine the effect of reduced levels of Hus1 on cell transformation, primary cells expressing incrementally reduced levels of Hus1 were tested for their ability to become immortalized, the first steps toward becoming transformed. Primary MEFs from the Hus1 allelic series were transfected with a plasmid expressing Large T antigen, a potent oncogene, and plated at low density. Immortalization capability was assessed by the number of colonies that formed (Fig. 2). Cells expressing reduced levels of Hus1 were less readily immortalized than cells expressing wild-type levels of Hus1. These preliminary data suggest that cells with reduced levels of Hus1 cannot undergo rapid proliferation and expansion to form a colony following expression of a potent oncogene, Large-T antigen. This assay will be repeated in additional primary cell cultures using a more efficient, viral method to express Large-T antigen.
  - c. **Focus formation assay:** In order to determine the effect of reduced levels of Hus1 on cell transformation, immortalized cells expressing incrementally reduced levels of Hus1 were tested for their ability to overcome contact inhibition, a hallmark of transformation. Primary and immortalized MEFs from the Hus1 allelic series were transfected with expression plasmids encoding activated H-Ras alone, activated H-Ras and E1A, or E1A alone and tested for transformation as measured by focus formation, which is a result of a cell's overcoming contact inhibition and continuing to proliferate. Three independently derived immortalized cultures were used for these experiments. In general, immortalized MEF cell lines expressing the lowest level of Hus1 showed fewer foci than control MEF cell lines following transfection with activated H-Ras and E1A (Fig. 3). Though these focus assays were consistent within each cell line, there was variability between cell lines. This is probably due to other underlying mutations which have accumulated during the immortalization process. However, the general trend of these focus assays indicates that cells with reduced levels of Hus1 are less prone to euplastic transformation following oncogenic signaling, resulting in a reduced number of focus formation. This assay will be repeated using cell lines immortalized by expression of Large-T antigen, rather than spontaneous immortalization in order to avoid the variability between cell lines due to additional underlying mutations.
  - d. **Anchorage independent growth assay:** In order to determine the effect of reduced levels of Hus1 on cell transformation, immortalized cells expressing incrementally reduced levels of Hus1 were tested for their ability to undergo anchorage independent growth, another indicator of malignant transformation. Immortalized MEFs from the Hus1 allelic series were infected with viruses expressing GFP, activated H-Ras, or both activated H-Ras and c-Myc and grown in soft agar to determine anchorage independent growth potential. Three independently derived, immortalized cells lines were used in this assay. Two of

- three Hus1<sup>+</sup> cell lines developed a large number of colonies (148 and 185) following addition of Ras and Myc, while one Hus1<sup>+</sup> cell line developed very few colonies (14), suggesting, on average, Hus1<sup>+</sup> cells are readily transformed after addition of two active oncogenes. All three Hus1<sup>Neo/Neo</sup> cell lines showed reduced colony formation relative to Hus1<sup>+</sup> cells after infection with Ras and Myc (10, 33, 57). Two of three Hus1<sup>Neo/ $\Delta$ 1</sup> cell lines developed reduced colony numbers compared with Hus1<sup>+</sup> cell lines following infection with Ras and Myc (33 and 52). The remaining Hus1<sup>Neo/ $\Delta$ 1</sup> cell line developed more than twice as many colonies (357) as Hus1<sup>+</sup> cell lines; however, the resulting colonies were smaller than the colonies of Hus1<sup>+</sup> cells (Fig. 4). This suggests that cells with reduced levels of Hus1 that are able to be transformed are less able to proliferate to form large colonies in soft agar, as seen in Hus1<sup>+</sup> cells, following infection with active oncogenes. Taken together, Hus1<sup>Neo/Neo</sup> and Hus1<sup>Neo/ $\Delta$ 1</sup> cells more consistently show decreased transformation ability in comparison to Hus1<sup>+</sup> in this more stringent soft agar assay, as compared to a focus assay.
- e. Transplantation assay: In order to determine the effect of reduced levels of Hus1 on cell transformation, immortalized cells expressing incrementally reduced levels of Hus1 were tested for their ability to form tumors *in vivo*. MEFs from the Hus1 allelic series were infected with viruses expressing GFP or H-Ras and c-Myc. Infected cells were then injected subcutaneously in the flanks of wild-type mice, and tumor growth was monitored. A single immortalized cell line was tested in this assay. Cells with reduced levels of Hus1 developed into significantly smaller tumors than cells expressing a wild-type level of Hus1 (Fig. 5), indicating that reduced levels of Hus1 results in decreased transformation, resulting in smaller tumors.

Task 2. Determine the effects of reduced Hus1 levels of on mammary tumorigenesis in transgenic mice expressing activated oncogenes

- a. Mice expressing varying levels of Hus1 were interbred with transgenic mice expressing activated H-Ras in the mammary gland. These mice developed mammary and Hadrian gland tumors very quickly, within 4 weeks. Because of this short tumor latency, these mice could not be readily used for interbreeding to generate a tumor cohort. For this reason, we concluded that MMTV-Ras would not be a useful strain to determine the effects of reduced Hus1 levels on mammary tumorigenesis in mice, and instead have focused on a second mouse mammary tumor model as described below.
- b. Mice expressing varying levels of Hus1 and transgenic mice overexpressing ErbB2 in the mammary gland (MMTV-Neu) were interbred to generate a cohort of mice. These mice were then aged until tumor development (Fig. 6A) or to 20 months, and onset of tumor development was noted, as well as, tumor size and number upon dissection. Hus1<sup>+</sup>MMTV-Neu<sup>Tg</sup> mice developed more tumors than Hus1<sup>Neo/ $\Delta$ 1</sup>MMTV-Neu<sup>Tg</sup> mice. However, the MMTV-Neu transgene on a 129 background was only 30% effective at inducing a single mammary tumor after an extended 20 month latency (Fig. 6B), making it difficult to determine the contribution of reduced Hus1 on reduced mammary tumor development. Additionally, Hus1<sup>+</sup> mice were much heavier and had more adipose tissue than

Hus1<sup>Neo/ $\Delta$ 1</sup> mice on this strain background (Fig. 6C and D). Although this is an intriguing observation that deserves further investigation, it complicates our analysis of mammary tumorigenesis. Because increased adipose tissue has been shown to increase inflammation, and inflammation has been linked to cancer development, the resulting decreased tumor burden in Hus1<sup>Neo/ $\Delta$ 1</sup> mice may be due to decreased fat and inflammation rather than decreased levels of Hus1 directly. The results were further complicated due to an increased number of Hus1<sup>Neo/ $\Delta$ 1</sup> mice, with or without MMTV-Neu<sup>Tg</sup>, which died from uterine pathology, which may have prevented mammary tumor development (Fig. 6E). To clarify this, a third genetic model of mammary tumorigenesis was used, MMTV-PyMT transgenic mice, which expression the Polyoma Middle T Antigen oncogene specifically in the mammary epithelium. MMTV-PyMT<sup>Tg</sup> mice should develop mammary tumors with a shorter latency than MMTV-Neu<sup>Tg</sup> mice, avoiding the complications of mice of advanced age.

- c. Histopathological analysis of mammary glands: Tumors harvested from MMTV-Neu<sup>Tg</sup> mice and MMTV-PyMT<sup>Tg</sup> mice are subjected to histopathological analysis. The tumors have been fixed and paraffin embedded, and will be analyzed by H&E staining. Immunohistochemistry will also be performed for markers of DNA damage, such as  $\gamma$ -H2AX, markers of senescence, such as p16, apoptosis by TUNEL staining, and proliferation by Ki67 staining.

#### Key Research Accomplishments:

- Primary MEFs expressing reduced levels of Hus1 were less able to be immortalized by transfection with a plasmid expressing Large-T antigen
- Three separately derived, spontaneously immortalized cell lines were established for each decreasing level of Hus1 expression, Hus1<sup>+</sup>, Hus1<sup>Neo/Neo</sup>, Hus1<sup>Neo/ $\Delta$ 1</sup>
- Focus formation assays using these established, immortalized cell lines have shown a trend that cells with reduced levels of Hus1 develop fewer foci following transfection with plasmids encoding two oncogenes, suggesting that the reduced levels of Hus1 interfere with neoplastic proliferation.
- Soft agar assays using these established, immortalized cell lines have shown a trend that cells with reduced levels of Hus1 develop fewer colonies following infection with viruses expressing two oncogenes, further suggesting that the reduced levels of Hus1 interfere with neoplastic proliferation.
- Transplantation assays using these established, immortalized cell lines show that cells with reduced levels of Hus1 develop smaller tumors following infection with viruses expressing two oncogenes and subcutaneous injection, suggesting that the reduced levels of Hus1 interfere with neoplastic proliferation, and that these results are more consistent in a more stringent assay.
- A cohort of MMTV-Neu expressing mice consisting of an equal number of mice with reduced levels of Hus1 and control (Hus1<sup>+</sup>) mice are being evaluated for differences in mammary tumor incidence and latency.
- A cohort of MMTV-PyMT expressing mice consisting of an equal number of mice with reduced levels of Hus1 and control (Hus1<sup>+</sup>) mice are being generated to determine differences in mammary tumor incidence and latency.

#### Reportable Outcomes:

##### Meeting Attended:

John B. Little Symposium, 12<sup>th</sup> Annual Symposium, “Stress responses in radiobiology, cancer and aging.”

##### Manuscript:

Yazinski, S.A., Westcott, P.M., Ong, K., Pinkas, J., Peters, R.M., and Weiss, R.S. (2009). Dual inactivation of Hus1 and p53 in the mouse mammary gland results in accumulation of damaged cells and impaired tissue regeneration. *Proc Natl Acad Sci U S A* 106, 21282-21287

#### Conclusions

In order to determine the effect of reduced levels of Hus1 on transformation, focus formation assays and soft agar assays in immortalized cells have been performed. These assays revealed a trend that, in general, cell lines with reduced levels of Hus1 develop fewer transformed foci and fewer colonies following expression of two oncogenes than cell lines expressing wild-type levels of Hus1. Additionally, cells with reduced levels of Hus1 are less able to form a tumor when injected into the flank of wild-

type mice. Taken together, these data suggests that cells with reduced levels of Hus1 are more resistant to transformation than cells with at least one copy of wild-type Hus1 and that Hus1 may be required for cell survival following the stress of neoplastic proliferation.

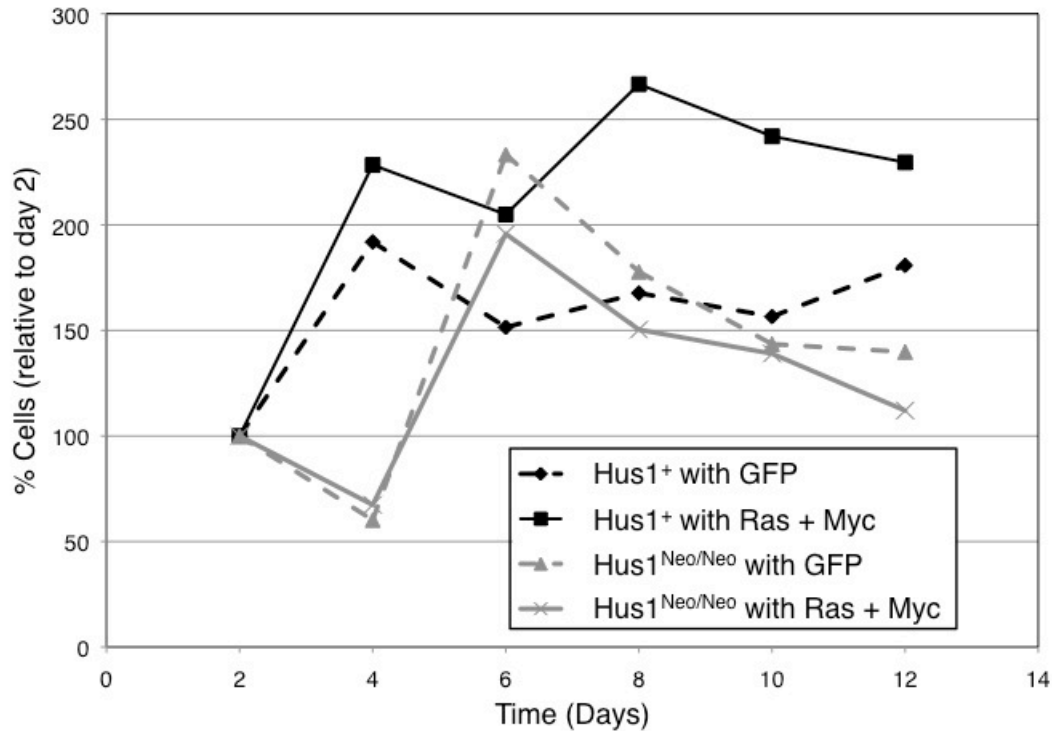
To test the effect of reduced levels of Hus1 on transformation and tumor development *in vivo*, a mouse model of mammary tumorigenesis, MMTV-Neu, was used. A cohort of mice with decreasing levels of Hus1 which overexpress an oncogene specifically in the mammary gland have been generated and evaluated for differences in mammary tumor development. There was no significant decrease in tumor development or change in latency between Hus1<sup>+</sup> and Hus1<sup>Neo/ $\Delta$ 1</sup> mice carrying the MMTV-Neu transgene. Because the MMTV-Neu model is only 30% effective at inducing mammary tumor development on this genetic background and there is a very long tumor latency, we will also begin investigating the effects of reduced levels of Hus1 on tumorigenesis using a third, more aggressive model of mammary tumorigenesis, MMTV-PyMT.

Taken together, preliminary data from the transformation assays suggest that cells require greater than 20% of wild-type Hus1 levels in order to efficiently undergo transformation. These results will be further tested in other cell culture assays using primary cells and in an *in vivo* mouse breast cancer model. The results of these studies will show whether Hus1, or the Atr pathway, may be a possible drug target for treatment of breast cancers.

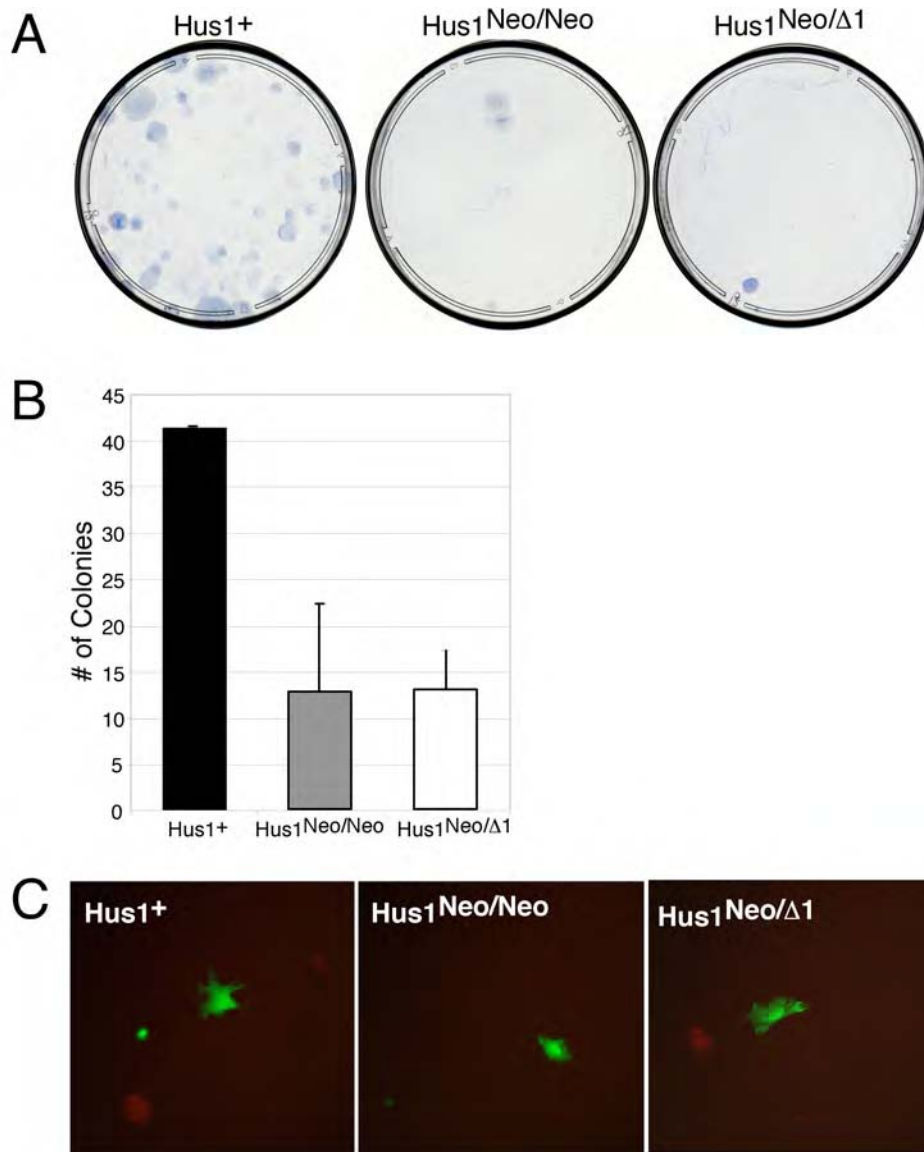
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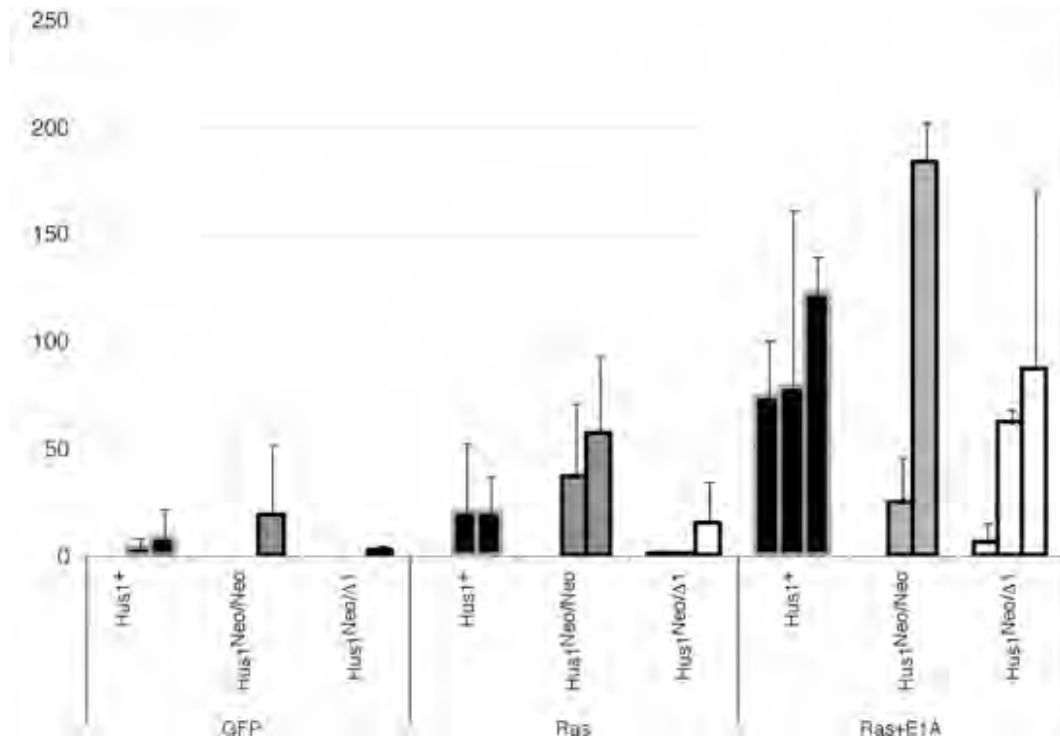
Supporting Data:



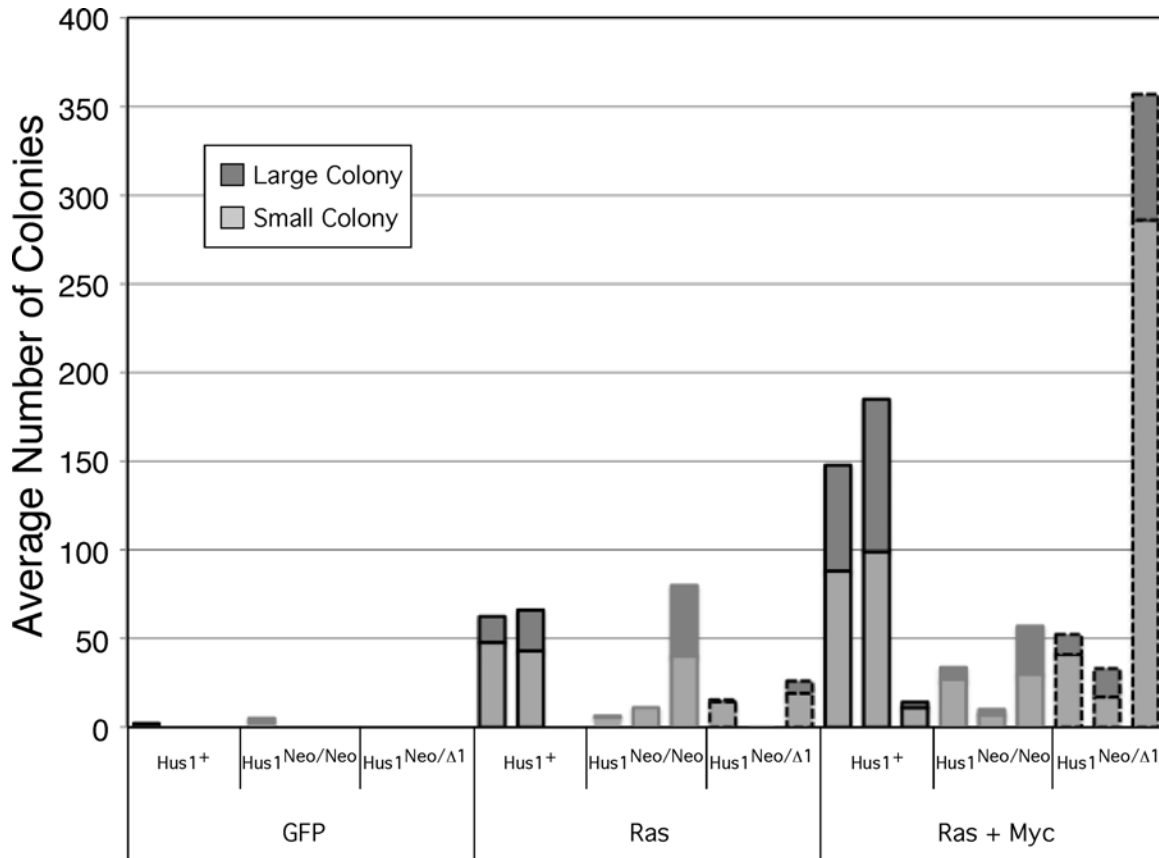
**Figure 1: MEFs expressing reduced levels of Hus1 show decreased proliferation following infection with a virus expressing two oncogenes.** Control Hus1<sup>+</sup> and Hus1<sup>Neo/Neo</sup> MEFs were infected with a virus expressing GFP or Ras and Myc, and surviving cells were counted every two days. Control Hus1<sup>+</sup> MEFs show an increase in cell proliferation following infection with a virus expressing Ras and Myc, relative to Hus1<sup>+</sup> cells infected with a virus expressing GFP. MEFs expressing reduced levels of Hus1 (Hus1<sup>Neo/Neo</sup>), on the other hand, show a decreased cell survival and proliferation in a short term proliferation assay following infection with a virus expressing two activated oncogenes (Ras and Myc) relative to Hus1<sup>Neo/Neo</sup> cells infected with a virus expressing GFP.



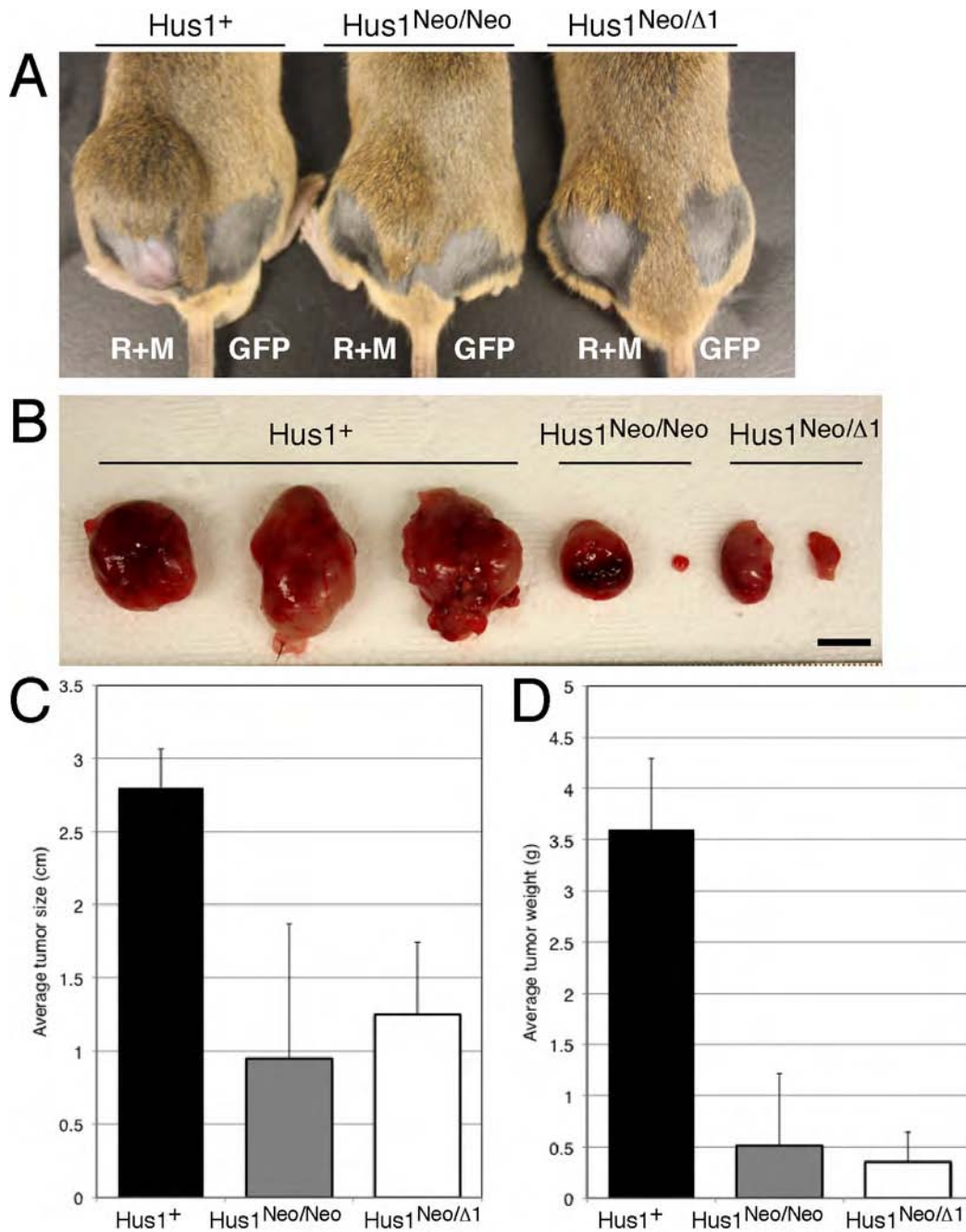
**Figure 2. Cells with reduced Hus1 expression are resistant to Large-T antigen induced immortalization.** (A) Representative images of primary mouse embryonic fibroblasts (MEFs) of the indicated genotypes two weeks after transfection with Large-T antigen. (B) Quantification of colony formation assay. Bars indicate the average of two independently derived averaged from colony formation at two densities ( $1 \times 10^3$  and  $1 \times 10^4$ ), with error bars indicating standard deviation. The number of colonies formed by Hus1<sup>+</sup> is not statistically different from Hus1<sup>Neo/Neo</sup> ( $p=0.194$ ) or Hus1<sup>Neo/ $\Delta$ 1</sup> ( $p=0.194$ ) by Students T-test.



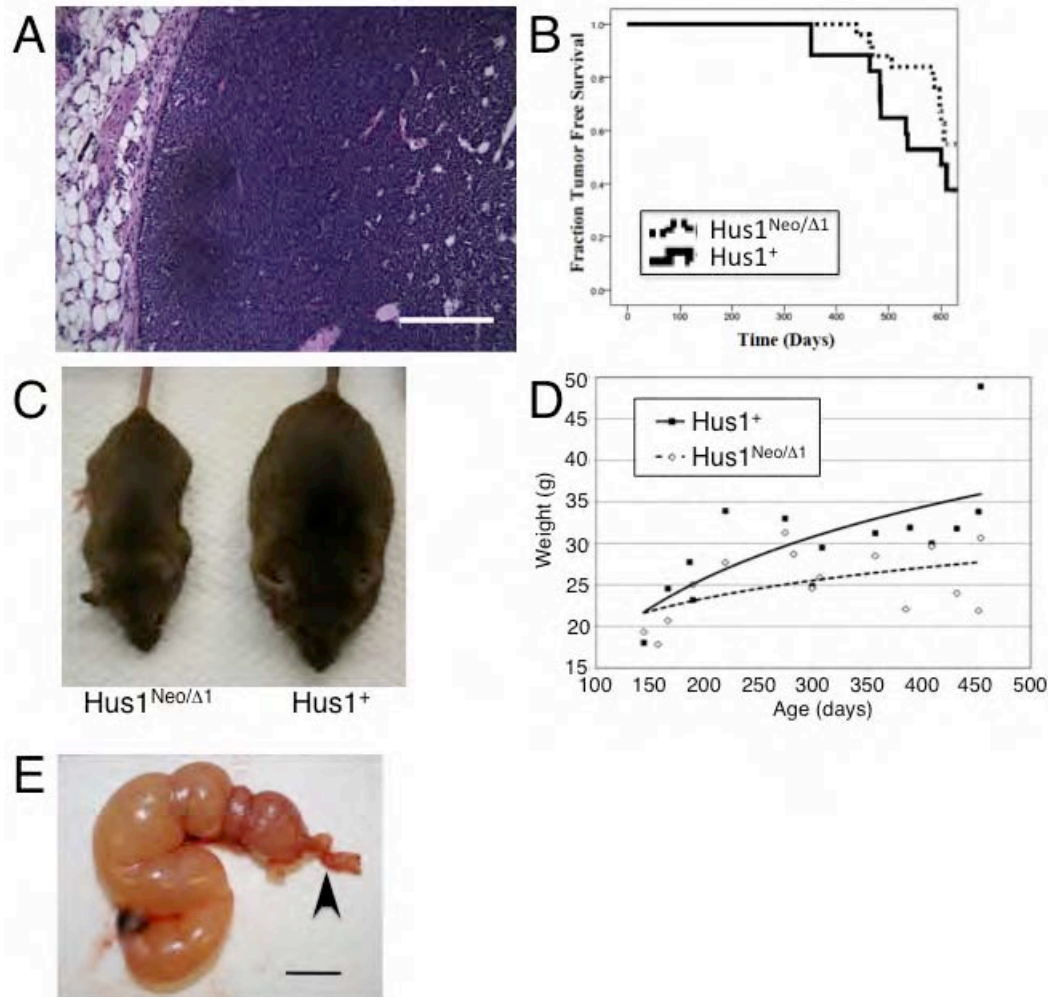
**Figure 3. Cells with reduced Hus1 expression show reduced focus formation in a contact inhibition transformation assay.** Three independently derived immortalized MEF cultures of the indicated genotypes were transfected with GFP, a single activated oncogene (Ras or E1A), or two activated oncogenes (Ras and E1A). Two weeks post transfection, cells were fixed with methanol and stained with Giemsa overnight. Foci were counted using ImageJ software. Bars indicate the average of at least two independent experiments on each of three cell lines, with error bars indicating standard deviation. There was no statistically significant difference in the average focus formation across cell lines following transfection with plasmids expressing Ras and E1A between Hus1<sup>+</sup> and Hus1<sup>Neo/Neo</sup> ( $p=0.727$ ) or Hus1<sup>Neo/Δ1</sup> ( $p=0.232$ ) by Students two-tailed T-test.



**Figure 4. Cells with reduced Hus1 expression have a decreased capacity to form colonies in an anchorage independent growth transformation assay.** Three independently derived immortalized mouse embryonic fibroblasts (MEFs) of the indicated genotypes were infected with viruses expressing GFP, a single activated oncogene (Ras), or two activated oncogenes (Ras and Myc) and grown in a soft agar assay. Resulting colonies were counted using ImageJ software. Bars indicate the average of at least two independent experiments on each of three cell lines.



**Figure 5. Transplantation of Hus1<sup>+</sup> cells infected with Ras and Myc results in larger tumors than transplantation of Hus1<sup>Neo/Neo</sup> and Hus1<sup>Neo/Δ1</sup> cells infected with Ras and Myc.** (A) Images of mice injected with Hus1<sup>+</sup>, Hus1<sup>Neo/Neo</sup>, or Hus1<sup>Neo/Δ1</sup> cells that were infected with a virus expressing GFP or expressing Ras and Myc. (B) Images of tumors resulting from injection with Hus1<sup>+</sup>, Hus1<sup>Neo/Neo</sup>, or Hus1<sup>Neo/Δ1</sup> cells that were infected with a virus expressing Ras and Myc. No tumors developed from cells of any genotypes infected with a virus expressing GFP. Scale bar represents 1cm. (C and D) (C) Average size and (D) average weight of tumors resulting from injection with Hus1<sup>+</sup> (n = 3), Hus1<sup>Neo/Neo</sup> (n = 2), or Hus1<sup>Neo/Δ1</sup> (n = 2) cells that were infected with Ras and Myc.



**Figure 6.  $Hus1^+$  MMTV-Neu $^+$  and  $Hus1^{Neo/\Delta 1}$  MMTV-Neu $^+$  mice develop mammary tumors, while  $Hus1^{Neo/\Delta 1}$  mice are leaner and develop uterine pathology, regardless of MMTV-Neu transgene status. (A) Representative H&E stained image of a MMTV-Neu induced mammary tumor from a  $Hus1^+$  MMTV-Neu $^+$  mouse. Scale bar represents 200 $\mu$ m. (B) Kaplan-Meier plot showing fraction of tumor free survival over time for all  $Hus1^+$  MMTV-Neu $^+$  and  $Hus1^{Neo/\Delta 1}$  MMTV-Neu $^+$  mice. Mice were euthanized when a mammary tumor developed, when uterine pathology developed, or at 600 days. There is no statistically significant difference in tumor free survival between  $Hus1^+$  MMTV-Neu $^+$  and  $Hus1^{Neo/\Delta 1}$  MMTV-Neu $^+$  ( $p=0.466$  by Log Rank survival test). (C) Image showing body size difference between  $Hus1^+$  and  $Hus1^{Neo/\Delta 1}$  mice on a mixed background. (D) Weight differences across age between  $Hus1^+$  and  $Hus1^{Neo/\Delta 1}$  on a mixed 129SvEv and FVB background. (E) Abnormal uterine pathology was found more frequently in  $Hus1^{Neo/\Delta 1}$  mice than  $Hus1^+$  mice, regardless of MMTV-Neu transgene status. Arrowhead indicates start of right uterine horn, which has grossly normal uterine morphology. Scale bar represents 1cm.**