Supplemental Data:

Methods

Soft Agar Colony Forming Assay

Stock agar and agarose solutions were prepared by dissolving agar (Difco, Sparks, MD) in Hank's balanced saline salt solution (Invitrogen, Carlsbad, CA) and agarose (Denville Scientific, Metuchen, NJ) in DMEM containing 13.3% FBS and autoclaving prior to use. Agar and agarose were cooled to 40°C in a waterbath, and agar was mixed with prewarmed DMEM to give a final agar concentration of 0.5%. This mixture was poured into 6 well tissue culture plates for the preparation of the base agar. Cultured mouse embryonic fibroblast cells were trypsinized and counted. Trypsinized cells were diluted to a volume that would yield 5,000 cells per plate and cells were mixed with cooled agarose to yield a final agarose concentration of 0.7%. Triplicate plates were prepared for each cell line. Plates were incubated at 37°C with 5% CO2 for 14 days. Following incubation, plates were stained with 0.5 ml of 0.005% crystal violet (Sigma, St. Luis, MO), and colonies were counted using a dissecting microscope.

Flow cytometric micronucleus assay

Transgenic, wildtype, and radiation sensitive positive control NOD/SCID mice aged 6-8 weeks were subjected to either 0.7 Gy or 2 Gy of γ-irradiation from a ^{137}Cs source. Fourty-eight hours later, 50ml of peripheral blood was collected from the retro-orbital sinus into a tube containing 250ml of anticoagulant solution (500U/ml heparin in PBS). Treated peripheral blood (180 ml) was transferred to a polypropylene centrifuge tube containing 2ml of methanol at -80c. Tubes were struck sharply several times to break up any clumps and stored at least overnight at -80c until further processing.

Fixed blood samples were stained with FITC-conjugated anti-CD71 (transferring receptor) antibody (Biodesign, Saco, ME) to identify mature erythrocytes. Prior to analysis by flow cytometry, samples were incubated in 1mg/ml propidium iodide solution to stain nuclei. Flow cytometric analysis was performed on a BD Coulter EPICS XL flow cytometer (BD Biosciences, San Jose, CA) at an excitation range of 488 nm, and a 620 band-pass filter for PI. At least 20,000 events were analyzed per sample, and each sample was run in triplicate, with three mice sampled from each group.

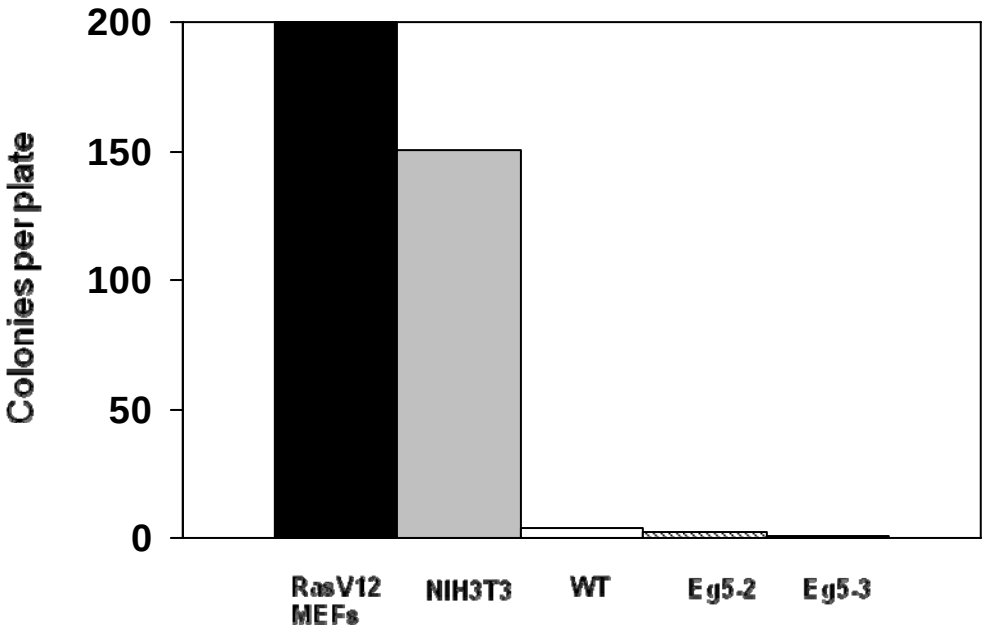
Immunoblotting

Equal amounts of cell lysate were resolved by SDS-PAGE and blotted to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membrane was blocked in 5% milk in TBS-Tween 20 [TBST; 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] for 1 hour at room temperature and incubated overnight at 4°C with polyclonal Eg5 antibody diluted at 1.5 µg/ml in TBST containing 2% dry milk. Membranes were washed 3 times with TBST and then incubated in HRP-conjugated anti-rabbit secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) diluted at 1:10,000 in TBST containing 2% dry milk for 1 hour. After additional washes, enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech, Piscataway, NJ) were applied, and blots were exposed to ECL-sensitive film.

Results

Soft Agar Colony Forming Assay

To determine if overexpression of Eg5 in cultured MEFs results in transformation and anchorage independent growth, soft agar colony forming assays were performed. Results from these studies showed no significant increase in the number of colonies between wildtype and transgenic MEFs following 14 days of incubation (S. Figure 1). Both control NIH-3T3 and RasV12 transduced MEFs displayed significantly higher levels of anchorage independent colonies. These data in conjunction with previous data from looking at Eg5's transforming properties, suggest that overexpression of Eg5 is not sufficient for transformation in cultured MEFs.



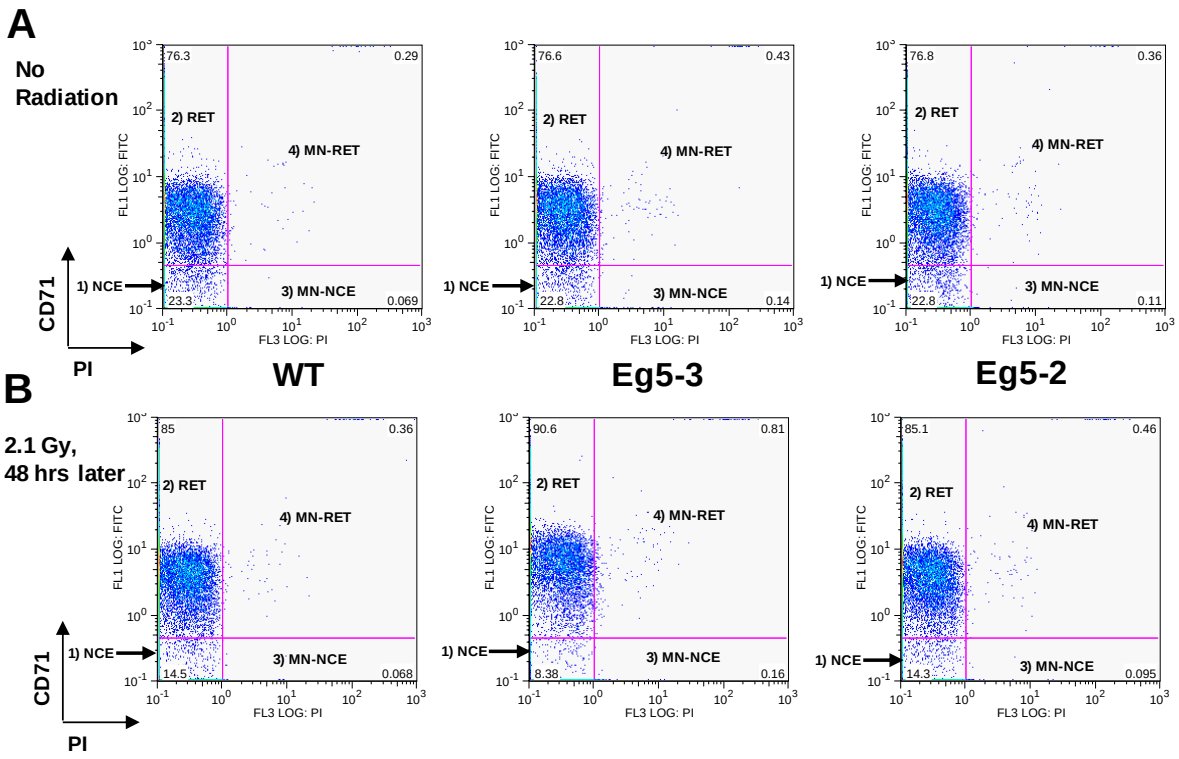
Castillo, A. – Supplemental Figure 1. 5,000 cells per line were cultured for 2 weeks on 0.7% agarose and monitored for the formation of colonies. Positive controls RasV12 transduced MEFs and NIH3T3s displayed a significant number of colonies, while WT and transgenic (Eg5-2 and Eg5-3) MEFs displayed little or no colonies. Triplicate plates were prepared for each line (averages shown) and counted following crystal violet staining.

Micronuclei Assay for Chromosome Instability

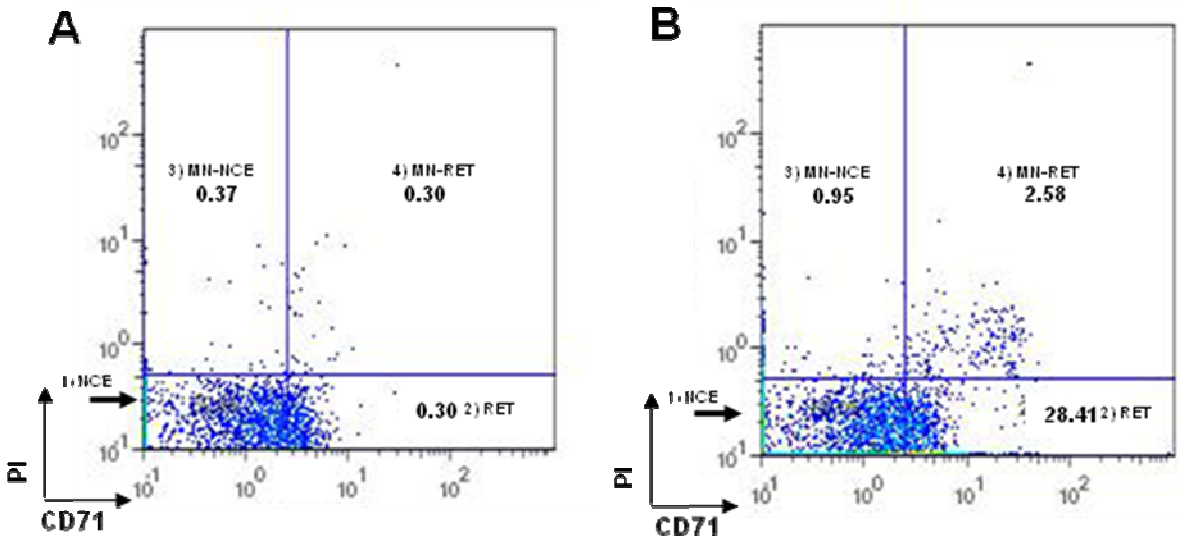
To detect chromosomal missegregation the high-throughput micronucleus assay was employed to detect whole chromosomes or chromosomal fragments that have missegregated. Following anaphase, displaced chromatin, which consists of whole chromosomes or acentric chromosomal fragments, can be excluded from the nuclei of daughter cells and is found in the cytoplasm as micronuclei. Flow cytometric analysis of erythrocytes has proven a useful technique in the identification of chromosome instability, as erythroblasts expel their nucleus, but not micronuclei, within a few hours following the last mitosis during erythrocyte maturation (1-3).

Anti-CD71 antibody was used to distinguish between normo-chromatic erythrocytes and reticulocytes, and micronuclei were visualized by propidium iodide staining of nucleic acid material. Transgenic and wildtype mice were initially treated with 0.7 Gy of γ-irradiation to induce DNA damage and micronuclei formation. Radiation sensitive *NOD/SCID* mice were used as positive controls and were subjected to similar doses of γ-irradiation. Forty-eight hours after irradiation, peripheral blood was analyzed by flow cytometry for the presence of micronuclei. At this dose of irradiation, no significant differences were found between wildtype and transgenic lines. Similarly, irradiation failed to induce micronuclei formation in positive control *NOD/SCID* mice.

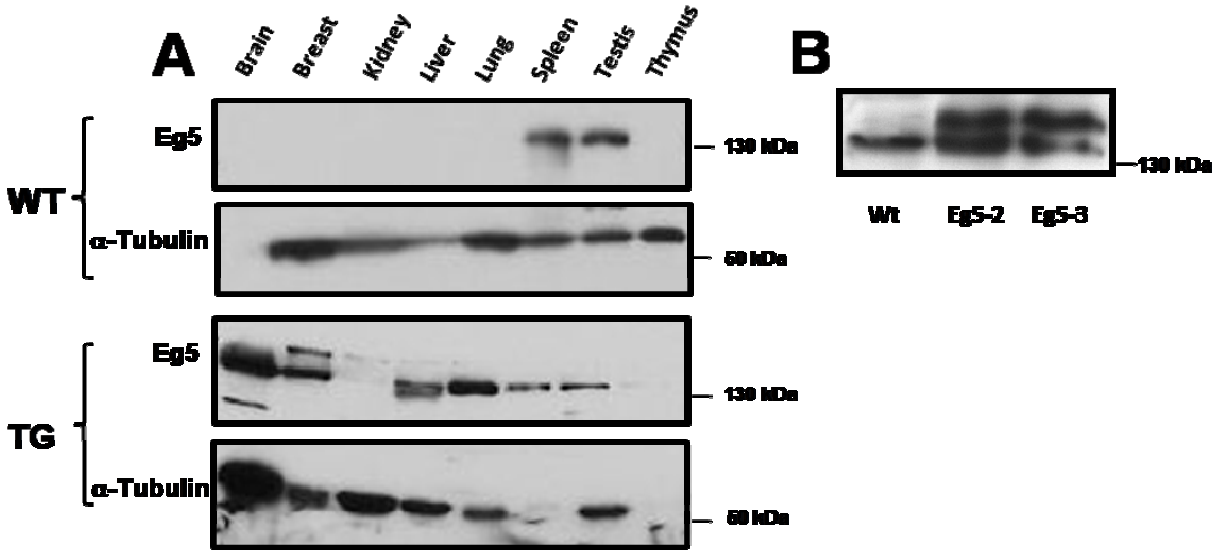
Higher doses of γ-irradiation were used to induce micronuclei formation as lower doses failed to induce a significant increase in the rate of micronuclei in both sample subsets and positive control *NOD/SCID* mice. Transgenic, wildtype, and *NOD/SCID* mice were treated with 2.1 Gy of γ-irradiation and peripheral blood was collected 48 hrs later. At this dose of radiation induced DNA damage, both wildtype and transgenic samples displayed only minimal increases in rates of micronuclei induction (S. Figure 2). Positive control SCID mice however did display a significant increase in irradiated samples (S. Figure 3).



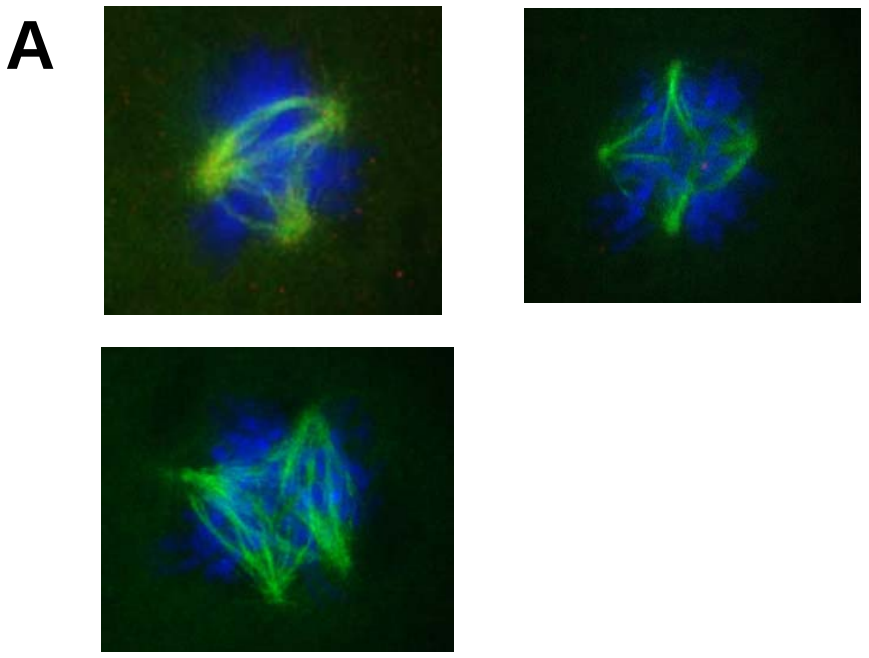
Castillo, A – Supplemental Figure 2. Peripheral blood from Pim1-Eg5 (PE2 and PE 3) mice before (A) and 48hr after (B) γ-irradiation (2.1 Gy) was analyzed. CD71 positive reticulocytes (populations 2 and 4) are separated from CD71 negative normo-chromatic erythroblasts (populations 1 and 3) to enumerate radiation-induced micronuclei (MN). MN are stained were stained with propidium iodide (PI) and shown as populations 2 and 4.



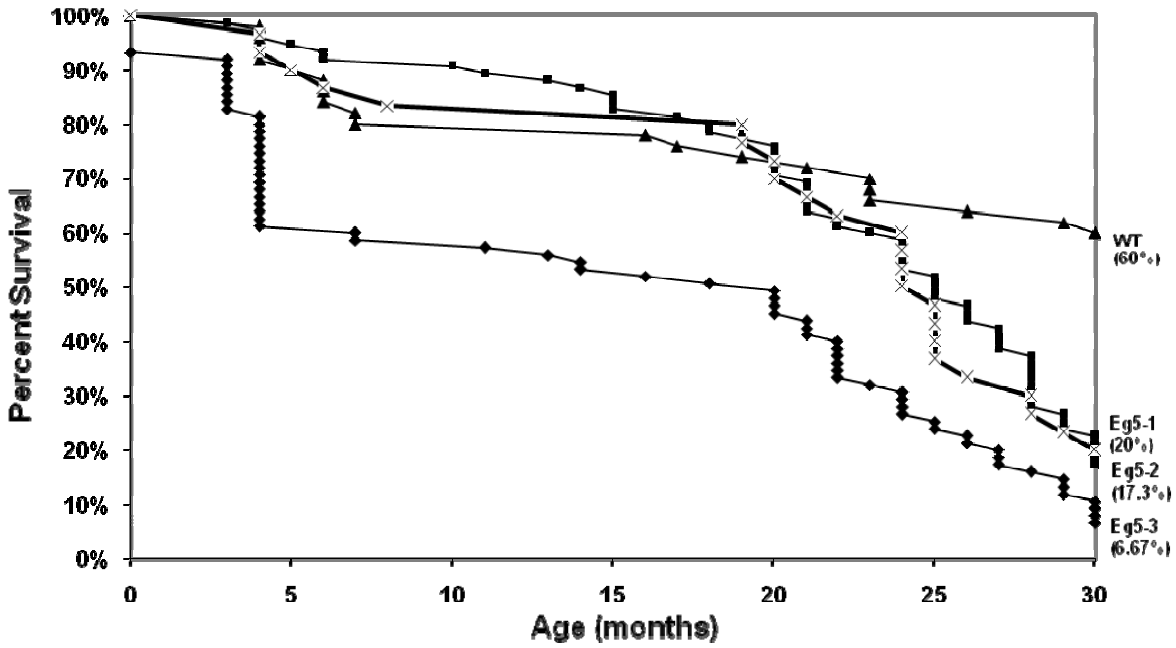
Castillo, A – Supplemental Figure 3. Micronuclei quantitation by flow cytometric analysis. Peripheral blood from NOD/SCID positive control mice before (A) and 48hr after (B) γ-irradiation (2.1 Gy) was analyzed. CD71 positive reticulocytes (populations 2 and 4) are separated from CD71 negative normo-chromatic erythroblasts (populations 1 and 3) to enumerate radiation-induced micronuclei (MN). MN are stained were stained with propidium iodide (PI) and shown as populations 2 and 4.



Castillo, A – Supplemental Figure 4. Western blot analysis of protein expression in transgenic mice and MEFs. Analysis was performed on cell lysates extracted from multiple mouse tissues and cultured MEFs using a polyclonal antibody to the linker domain of Eg5. (A) A single band could be identified migrating at ~130kDa in samples collected from tissues, (B) while two bands were observed in cultured transgenic MEFs, representing both unphosphorylated (lower band) and mitotically active phosphorylated (upper band) forms of Eg5 (4). Two phosphorylation sites have been reported for Eg5. One resides in the α-helical stalk domain that is acted upon by the aurora kinase, STK15. A second phosphorylation site lies in the bim-C box located in the tail domain, and phosphorylation by $p34^{cdc2}$ protein kinase at this site is required for localization to the mitotic spindle (5, 6). In wildtype tissues, Eg5 was detected in spleen and testis samples only, while in transgenic line Eg5-3 protein was detectable in all tissues.



Castillo, A – Supplemental Figure 5. Additional Images of multipolar and monopolar spindles observed in transgenic MEFs. (A) Cells containing from 3 to 5 spindle poles were observed at a significantly higher frequency in transgenic MEFs compared to wildtype. Microtubule visualized in green (α-tubulin) and DNA in blue (DAPI). (B) Monopolar spindle observed in transgenic MEFs with microtubules, shown in red (α-tubulin), radiating outward from unseparated spindle poles. (C) γ-tubulin staining of centrosomes. Two distinct foci are visible in biopolar spindles formed in transgenic MEFs.



Castillo, A. – Supplemental Figure 6. Survival curves for wildtype FVB/NJ and *Tg(Eg5)Jus* transgenic mice. Time of death was recorded due to cancer and/or additional phenotypes for wildtype and three lines of *Tg(Pim1-Eg5)Jus* mice. Transgenic mouse survival was significantly reduced (P<0.02, Eg5-1; P<.001, Eg5-2; P<0.0001, Eg5-3 as tested by log-rank test) compared to wildtype FVB/NJ mice (n=50, WT; n=30, Eg5-1; n=70, Eg5-2; n=50, Eg5-3). Additional phenotypes consisted of neurological abnormalities, megacystis, and dermatitis.

Matings	Tg (+/-) -Eg5 (+/-) X Tg (+/-) -Eg5 (+/-)		
Expected Genotypes	[Tg (+/+) -Eg5 (+/+) Tg (+/-) -Eg5 (+/+)]	[Tg (+/+) Eg5 (+/-) Tg (+/-) -Eg5 (+/-)]	[Tg (+/+) Eg5 (-/-) Tg (+/-) -Eg5 (-/-)]
Observed Genotypes	**29**	**82**	**3**

Castillo, A – Supplemental Table 1. Transgenic rescue of Eg5$^{-/-}$ early embryonic lethality

References for Supplemental Data

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