context of segregating variation allows the discovery of subtle effects obscured in strains with engineered knockouts.

We have found that regulatory genetic variation is characterized by a high rate of cis-acting alleles and a small number of trans-acting alleles with widespread transcriptional effects. Finally, genetic variation in physiological and behavioral quantitative phenotypes is known to be highly complex. Our results indicate that even in a single-cell organism grown in a controlled environment, variation in gene expression typically also has a polygenic basis.

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8. Parent strains were BY4716, an S288c derivative (*MATa lys2Δ0*) (*27*), and RM11-1a (*MATa leu2Δ0 ura3Δ0 HO:kan*), a haploid derived from Bb32(3), a natural isolate collected by Robert Mortimer, as in (*28*). Auxotrophic deletions were used to select diploids during mating and as positive controls for linkage. Both strains were gifts from Lee Hartwell, FHCRC, Seattle, WA.
9. Cultures were grown to 1×10^7 cells/mL in synthetic C medium at 30°C and shaken at 175 rpm in a shaker bath. RNA was isolated with hot acid phenol and chloroform (*29*), followed by the RNA cleanup protocol for RNeasy columns (Qiagen, Valencia, CA). Samples were labeled and hybridized to cDNA microarrays, containing 6215 open reading frames, as described (*30*). Images were analyzed with GenePix software. Each two-color experiment involved one sample and one reference, with the same BY4716 RNA reference being used for all experiments. Two hybridizations were carried out for each sample, one with the sample labeled with Cy3 and the reference with Cy5, and one with the fluors reversed; for each gene, the two log ratios were averaged.
10. Differences in the expression between parent strains were assessed by comparing the six RM values to the six BY values with the Wilcoxon-Mann-Whitney test (*31*), and nominal *P* values are reported. To estimate the number of differences expected by chance at a given *P*-value threshold, we randomly assigned 6 of the 12 measurements to one group and 6 to the other, without regard for strain identity. We then compared the two groups, using the same statistical test we applied in comparing the two strains, and recorded how many genes had *P* values below the threshold. The randomization procedure was repeated 100 times, and the results were averaged.
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21. For each message differentially expressed between the parents at $P < 0.005$, we computed the expression difference, *x*, in units of standard deviation. We simulated a locus explaining a fraction *f* of this difference by randomly assigning parent genotypes at the locus for 40 segregants and drawing the expression level of each segregant from one of two Gaussian distributions of unit variance, one with mean 0 and the other with mean *fx* conditional on the genotype. We recorded the proportion *q* of messages for which linkage was detected at $P < 5 \times 10^{-5}$ in the simulated data, averaged over 100 simulations. In the text, $q = 97\%$ for $f = 1$, and $q = 29\%$ for $f = 1/3$. For *n* loci, each explaining a fraction $f = 1/n$ of the difference *x*, we computed the rate of detecting at least one locus as $1 - (1 - q)^n$.
22. The genome was divided into 611 bins of 20 kb each (the bins at ends of chromosomes were smaller). Excluding self-linkages, we found 385 linkages at $P < 5 \times 10^{-5}$. If these were randomly distributed across the genome, the number of linkages in any one bin would follow a Poisson distribution with a mean of 0.63. The probability that the bin with the most linkages would contain >5 is <0.03.
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Transcription Control by E1A and MAP Kinase Pathway via Sur2 Mediator Subunit

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Sur2 is a metazoan Mediator subunit that interacts with the adenovirus E1A protein and functions in a mitogen-activated protein kinase pathway required for vulva development in *Caenorhabditis elegans*. We generated *sur2*$^{-/-}$ embryonic stem cells to analyze its function as a mammalian Mediator component. Our results show that Sur2 forms a subcomplex of the Mediator with two other subunits, TRAP/Med100 and 95. Knock-out of Sur2 prevents activation by E1A-CR3 and the mitogen-activated protein kinase–regulated ETS transcription factor Elk-1, but not by multiple other transcription factors. These results imply that specific activation domains stimulate transcription by binding to distinct Mediator subunits. Activation by E1A and Elk-1 requires recruitment of Mediator to a promoter by binding to its Sur2 subunit.

Regulation of transcription by RNA polymerase II (Pol II) is controlled by specific combinations of sequence-specific, DNA binding regulatory transcription factors (TFs) bound to a gene's promoter and enhancer regions. These TFs interact with several multiprotein complexes that remodel the chromatin context of the promoter, integrate signals from multiple TFs to control the frequency of transcription initiation, and regulate the efficiency of transcription elongation (1). One such complex is the Mediator, an ~2-megadalton complex of 20 to 30 subunits that is believed to function as a molecular bridge by simultaneously interacting with both DNA-bound TFs and Pol II (*1–4*). The human Mediator subunit Sur2 was identified through its interaction with adenovirus E1A conserved region 3 (E1A-CR3), a potent activation domain that regulates early adenovirus genes (*5*). In *C. elegans*, mutations in *sur2* suppress an activated-*ras* multivulva phenotype and produce

multiple pleiotrophic developmental defects (*6*). Sur2 was shown to interact genetically with a receptor tyrosine kinase (RTK)–Ras–mitogen-activated protein kinase (MAPK)–regulated ETS DNA binding-domain (DBD) TF, Lin-1. The ETS family of TFs functions as critical nuclear targets of RTK-Ras-MAPK signal transduction pathways in metazoans, regulating cell proliferation, differentiation, and survival (*7*). Their associated activation or repression domains are regulated by phosphorylation by MAPKs.

We generated *sur2*$^{-/-}$ murine embryonic stem (ES) cells by successive homologous recombination of constructs that substituted different drug resistance cassettes for the second through fourth exons at each *sur2* locus (*8*). Mediator from *sur2*$^{-/-}$ ES cells was similar in size to the complex from wild-type (wt) ES cells. It fractionated similarly to human Mediator (*9*) during gel filtration of nuclear extract and immunoblotting with antibodies to several Mediator subunits (*10*). We partially purified Mediator from wild-type and *sur2*$^{-/-}$ ES cells by immunoprecipitation using an antibody against CDK8 (Fig. 1). In addition to the absence of Sur2, a band of ~100 kD was significantly reduced in *sur2*$^{-/-}$ cell Mediator. Mass spectrometry (*8*) identified the reduced ~100-kD band as murine Med100. Western blotting showed that TRAP/Med95 was also reduced in the *sur2*$^{-/-}$ Mediator. These results suggest that the mammalian Mediator contains a Sur2 subcomplex that includes Sur2, Med100, and Med95. In the absence of Sur2, Med100 and Med95 are less stably bound to the complex and are partially lost either in vivo or during purification. Consistent with this result, Mediator purified from murine cells with a knockout of TRAP/Med100 also has reduced Sur2 and TRAP/Med95 (*11*). Total Med95 was not reduced in *sur2*$^{-/-}$ nuclear extract but was largely monomeric (*10*). Med100 could not be analyzed because of poor cross-reactivity with an antibody against human TRAP100.

We analyzed several activation domains in *sur2*$^{-/-}$ cells in transient transfection assays using Gal4 DBD-activation domain fusions. Whereas VP16 activated equivalently in wild-type and *sur2*$^{-/-}$ cells (Fig. 2A), E1A-CR3 was completely defective in the mutant cells (Fig. 2B). Addition of a human Sur2 expression vector rescued the E1A defect in the mutant cells, demonstrating that the only missing functions required for E1A activation in *sur2*$^{-/-}$ cells are supplied by Sur2 (Fig. 2B). To test the ability of E1A-CR3 to activate transcription in the context of a viral infection, we assayed expression of early region 2 (E2). Although human adenovirus 2 (Ad2) does not infect murine ES cells as efficiently as HeLa cells, E2 RNA was detected after infection of wild-type ES cells with wild-type Ad2 and mutant pm975, expressing only the large E1A protein containing CR3, but not with dl1500, expressing only the small E1A protein lacking CR3 (*12*) (Fig. 2C). Consistent with the transfection results, E2 expression in the *sur2*$^{-/-}$ ES cells was severely diminished. This was not due to a difference in the efficiency of infection, because a recombinant adenovirus-expressing luciferase from the CMV-IE promoter produced somewhat more luciferase in the *sur2*$^{-/-}$ than the wild-type cells (*13*).

We used an immobilized template assay to analyze recruitment of Mediator from wild-type and *sur2*$^{-/-}$ nuclear extract to a promoter with bound Gal4–E1A-CR3 or Gal4-VP16 (Fig. 2D). Although TATA-binding protein bound to all DNA fragments irrespective of the presence of a TATA box or bound activator, binding of wild-type Medi-

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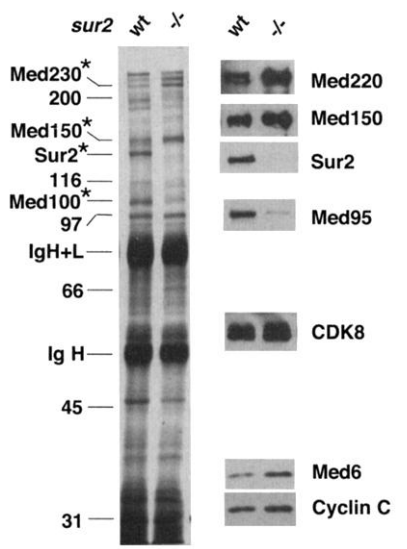

Fig. 1. Mediator from *sur2*$^{-/-}$ ES cells has reduced Med95 and Med100. Nuclear extract from wild-type (wt) and *sur2*$^{-/-}$ cells were immunoprecipitated with antibody against CDK8, subjected to SDS-PAGE and stained (left) or immunoblotted (right) with the indicated antibodies. Asterisk, stained bands identified by mass spectrometry. Numbers, MW markers. Methods for all figures (*8*).

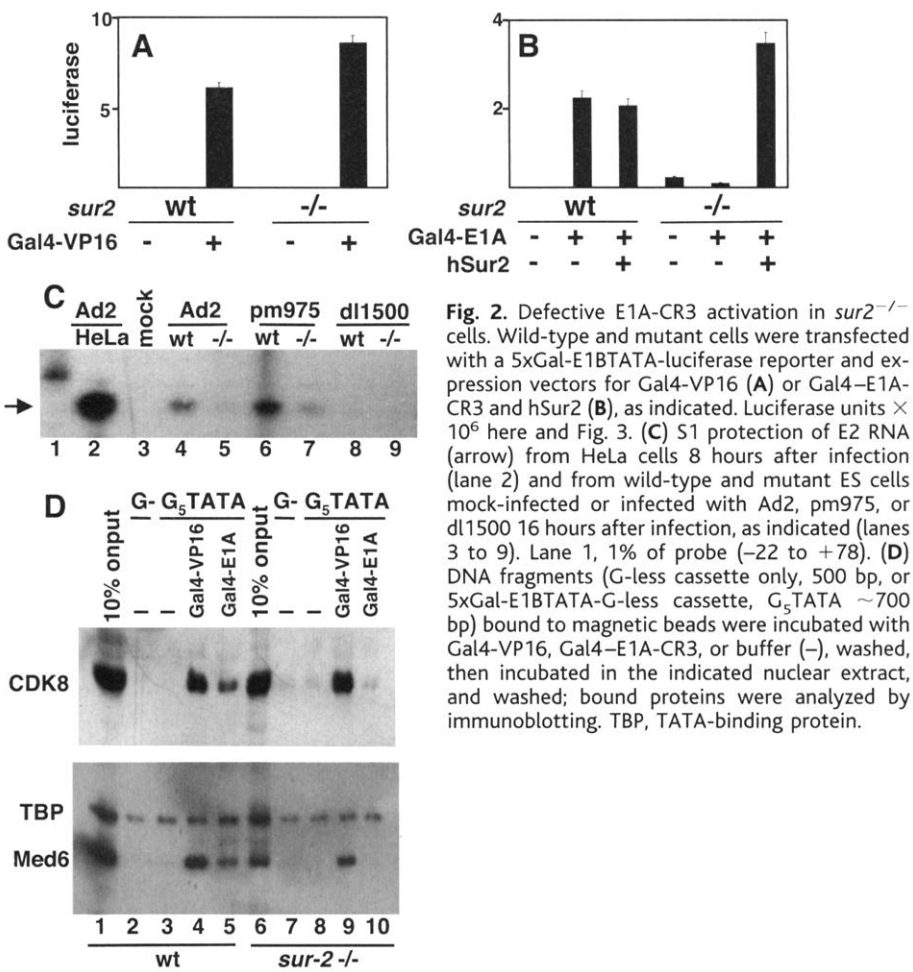

Fig. 2. Defective E1A-CR3 activation in *sur2*$^{-/-}$ cells. Wild-type and mutant cells were transfected with a 5xGal-E1BTATA-luciferase reporter and expression vectors for Gal4-VP16 (**A**) or Gal4–E1A-CR3 and hSur2 (**B**), as indicated. Luciferase units × 10^6 here and Fig. 3. (**C**) S1 protection of E2 RNA (arrow) from HeLa cells 8 hours after infection (lane 2) and from wild-type and mutant ES cells mock-infected or infected with Ad2, pm975, or dl1500 16 hours after infection, as indicated (lanes 3 to 9). Lane 1, 1% of probe (–22 to +78). (**D**) DNA fragments (G-less cassette only, 500 bp, or 5xGal-E1BTATA-G-less cassette, G_5TATA ~700 bp) bound to magnetic beads were incubated with Gal4-VP16, Gal4–E1A-CR3, or buffer (–), washed, then incubated in the indicated nuclear extract, and washed; bound proteins were analyzed by immunoblotting. TBP, TATA-binding protein.

ator, as assayed by immunoblotting for subunits CDK8 and Med6, required the addition of Gal4-VP16 or Gal4–E1A-CR3 (lanes 4 and 5). However, *sur2*$^{-/-}$ Mediator could only be recruited by Gal4-VP16, and not by Gal4–E1A-CR3 (lanes 9 and 10). We conclude that Sur2 is indeed the target for E1A-CR3 and that the recruitment of Mediator through an E1A-CR3–Sur2 interaction is required for E1A activation in vivo.

Based on genetic studies in *C. elegans* (*6*), we also assayed activation domains that are targets of MAPK signal transduction pathways. Elk-1, a member of the ternary complex factor (TCF) class of ETS-TFs that bind serum response elements (SREs) cooperatively with serum response factor (SRF) (*7*), and the b-zipper TF ATF-2 are both targets of MAPKs (*14*). Both are activated by an activated form of MEKK. Whereas activation by Gal4–ATF-2 was comparable in wild-type and *sur2*$^{-/-}$ cells in response to activated MEKK (Fig. 3A), activation by Gal4–Elk-1 was almost completely eliminated in *sur2*$^{-/-}$ cells (Fig. 3B). Similar results were observed when activated MEK1, a normal physiological activator of ERKs, was substituted for MEKK (*13*). As for E1A-CR3, activation by the Elk-1 activation domain in *sur2*$^{-/-}$ cells was restored by expressing hSur2 (Fig. 3B). As implied by this result, we found that the ERK2-phosphorylated Elk-1 activation domain, but not the unphosphorylated Elk-1 activation domain, bound Sur2 and/or Mediator, as indicated by detection of Sur2 in the bound fraction from HeLa nuclear extract (Fig. 3C). Gal4-Elk also recruited Mediator components in ES cell nuclear extract to a promoter in vitro, dependent on both phosphorylation by ERK2 and the presence of Sur2 (Fig. 3D).

Multiple other activation domains had comparable activity in wild-type and *sur2*$^{-/-}$ cells, including VP16 (Fig. 2A), ATF-2 (Fig. 3A), the ligand-binding domain of the glucocorticoid receptor activated by dexamethasone (Fig. 3E), ligand-binding domains of other nuclear receptors in response to their specific ligands (androgen receptor, PPARγ, LXRβ), p53, nuclear factor κB (p65), sterol response element–binding protein 1a, cyclic-AMP response element–binding protein in response to the catalytic subunit of protein kinase A, CHOP in response to MEKK, the Ewing sarcoma fusion protein (EWS), and the Zebra and Rta activators of Epstein-Barr virus. However, we observed a modest defect in activation by Ets-1 (Fig. 3F) and Ets-2 (*13*) in response to MEK1. Although ETS-1 and -2 are regulated by MAPKs, their activation domains are distinct from those of the TCFs such as Elk-1 (*7*). The modest ETS-1 and -2 defects were not rescued by coexpression of hSur2 (*13*).

We also examined the ability of the *sur2*$^{-/-}$ ES cells to initiate immediate early endogenous gene responses to serum mitogens. Immediate early genes such as *egr-1*, *egr-2*, c-*fos*, and c-*jun* show a rapid, transient increase in transcription after serum addition to serum-starved cells (*14*). *Egr-1* and *egr-2* are controlled by multiple SREs via signaling through the RTK-Ras-Raf-MEK-ERK pathway, dependent on TCFs (*15*–*17*). The c-*jun* promoter, on the other hand, does not contain SREs or ETS-binding sites (*14*). The c-*fos* promoter contains a single SRE, and binding of TCFs is only partially responsible for its serum activation (*18*, *19*). Serum induction of the endogenous *egr-1* and *egr-2* genes was dramatically depressed in *sur2*$^{-/-}$ cells (Fig. 4, A and B). In contrast, the c-*jun* serum response was normal, and activation of c-*fos* was only partially reduced. These results are in perfect agreement with earlier studies and our finding that, of multiple cellular activation domains tested, only the Elk-1 activation domain depends on Sur2. When wild-type cells were treated with the MEK-1–specific inhibitor U0126, c-*fos* induction was significantly inhibited, as expected (*20*) (Fig. 4C). In contrast, the induction in *sur2*$^{-/-}$ cells was much less sensitive to the drug, indicating that control of c-*fos* transcription was largely independent of the RTK-Ras-Raf-

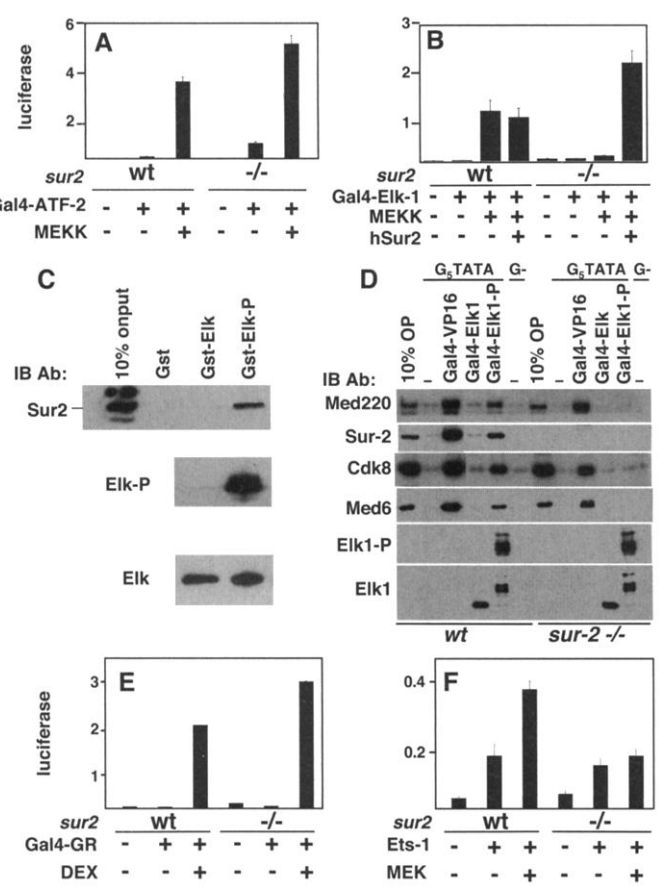

Fig. 3. Defective Elk-1 activation in response to MAPK signaling in *sur2*$^{-/-}$ cells. (**A**, **B**, and **E**) Wild-type and *sur2*$^{-/-}$ cells were transfected with a 5xGal-E1BTATA-luciferase reporter and expression vectors for activated MEKK, hSur2, and Gal4 DBD fusions to the indicated activation domains or the glucocorticoid receptor ligand-binding domain and treated with dexamethasone (DEX) as indicated. (**C**) HeLa nuclear extract was incubated 10 min at 30°C with matrix bound Gst, Gst-Elk, or Gst-Elk treated with activated Erk2 and ATP (Gst-Elk-P). After washing, eluted proteins were immunoblotted with the indicated antibodies. (**D**) Promoter recruitment as in Fig. 2D, using Gal4-Elk and Gal4–Elk-P. (**F**) Transfection of luciferase reporter with two upstream ETS-binding sites and expression vectors for Ets-1 and activated MEK, as indicated.

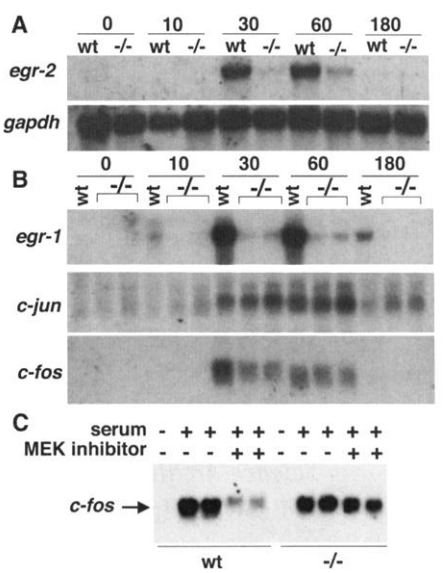

Fig. 4. Defective serum induction of specific immediate early genes in *sur2*$^{-/-}$ cells. (**A** and **B**) Northern blots of RNA isolated at the indicated minutes after serum addition to serum-starved wt or mutant cells, as indicated. In (B), two sister *sur2*$^{-/-}$ clones were analyzed in parallel. (**C**) Duplicate cultures of serum-starved cells were treated or untreated with 10 μM U0126 for 30 min; serum was added as indicated for 60 min and c-*fos* RNA analyzed.

MEK-ERK pathway in the mutant cells. Probably as a consequence of the defect in immediate early gene induction by serum, *sur2*$^{-/-}$ ES cells replicated significantly more slowly than wild-type ES cells in medium with 15% serum. However, their growth rate was more similar to wild-type cells in medium with 0.2% serum (*8*). These results demonstrate that activation of endogenous immediate early genes in response to the RTK-Ras-Raf-MEK-ERK pathway is defective in *sur2*$^{-/-}$ cells because Elk-1, and possibly other closely related TCFs, must interact with the Sur2 Mediator subunit to activate transcription.

These studies demonstrate that a function of the Mediator Sur2 subcomplex is to mediate transcriptional activation in response to RTK-Ras-Raf-MEK-ERK signaling to the TCF class of Ets-domain TFs. Although knocking-out *sur2* results in decreased Med100 and Med95 in the Mediator complex (Fig. 1), the defect in activation by E1A-CR3 and Elk-1 probably results from the absence of Sur2 because expression of excess Sur2 in wild-type cells inhibits (squelches) E1A-CR3 and Elk-1 activation specifically (*5*). Moreover, E1A-CR3 binds directly to a monomeric form of Sur2 isolated from HeLa cells (*5*, *9*), and the Erk2-phsophorylated Elk-1 activation domain interacts with Sur2 (Fig. 3C). Activation by Ets-1 and -2 also was modestly reduced in the *sur2*$^{-/-}$ cells. Perhaps the inability of transient hSur2 expression to rescue the Est-1 and -2 defect is because their activation domains interact with Med100 or 95, whose association with the mutant Mediator may be impaired during the time course of a transient transfection assay.

Studies in yeast have suggested that distinct Mediator subunits function in the activation mechanisms of specific classes of activation domains (*1*, *2*). The results reported here extend this model to higher eukaryotes. Most activators functioned well in the *sur2*$^{-/-}$ cells. Only E1A-CR3 and Elk-1 were severely defective. The large Sur2 subunit (~150 kD) may also interact with other as-yet-uncharacterized activation domains. Some activation domains probably function to activate transcription at several steps in the transcription process. For example, nuclear receptors interact with SWI/SNF chromatin remodeling complexes and histone acetylase complexes, as well as Mediator complexes (*21*). Perhaps this is why even though the ligand-binding domains of nuclear receptors bind to the Mediator subunit TRAP/Med220 in a ligand-dependent manner, TRAP220$^{-/-}$ cells continue to exhibit some activation in response to nuclear receptors (*22*). The finding that activation by E1A-CR3 and Elk-1 is virtually eliminated in the absence of Sur2 may indicate that the principal mechanism of transcriptional control by these activation domains is through recruitment of the Mediator.

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