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ETV6/RUNX1 abrogates mitotic checkpoint function and targets its key player MAD2L1

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Abstract

Approximately 25% of childhood B-cell precursor acute lymphoblastic leukemia have an *ETV6/RUNX1 (E/R)* gene fusion that results from a t(12;21). This genetic subgroup of leukemia is associated with near-triploidy, near-tetraploidy, and trisomy 21 as rather specific types of secondary changes. Here, we show that, unlike various controls, E/R-expressing Ba/F3 clones acquire a tetraploid karyotype on prolonged culture, corroborating the assumption that E/R may attenuate the mitotic checkpoint (MC). Consistent with this notion, E/R-expressing diploid murine and human cell lines have decreased proportions of cells with 4N DNA content and a lower mitotic index when treated with spindle toxins. Moreover, both RUNX1 and E/R regulate mitotic arrest-deficient 2 L1 (*MAD2L1*), an essential MC component, by binding to promoter-inherent RUNX1 sites, which results in down-regulation of *MAD2L1* mRNA and protein in E/R-expressing cells. Forced expression of E/R also abolishes RUNX1-induced reporter activation, whereas E/R with a mutant DNA-binding site leads to only minor effects. Our data link for the first time E/R, MC, and MAD2L1 and provide new insights into the function of the E/R fusion gene product. Although tetraploidy is an almost exclusive feature of E/R-positive leukemias, its rarity within this particular subgroup implies that further yet unknown factors are required for its manifestation.

Keywords

ETV6/RUNX1; t(12;21); acute lymphoblastic leukemia; mitotic checkpoint MAD2L1; tetraploidy

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Conflict of interest

The authors declare no conflict of interest.

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Introduction

The t(12;21)(p13;q22) with its molecular counterpart, the *ETV6/RUNX1 (E/R)* (also known as *TEL/AML1*) gene fusion, characterizes approximately 25% of childhood B-cell precursor ALL cases. *E/R* is unique among the fusion genes involving *RUNX1*, as it is associated with acute lymphoblastic leukemias rather than myeloid leukemias (Speck and Gilliland, 2002). Accumulating evidence suggests that this fusion gene can initiate leukemia development, and although it is not sufficient to cause overt leukemia *per se*, it is necessary for its maintenance (Zelent *et al.*, 2004; Diakos *et al.*, 2007; Hong *et al.*, 2008). *E/R* encodes a chimeric protein that is composed of the N-terminal non-DNA-binding region of ETV6 and almost the entire RUNX1 protein. It retains the runt domain of RUNX1 that is required for DNA binding and heterodimerization and, therefore, also provides an essential function of *RUNX1* fusion genes (Hiebert *et al.*, 1996; Morrow *et al.*, 2007; Roudaia *et al.*, 2009; Wolyniec *et al.*, 2009). The E/R protein acts as an aberrant transcription factor and represses or disrupts the regulation of RUNX1 target genes in a cellular context-dependent manner (Pui *et al.*, 2004; Zelent *et al.*, 2004; Wotton *et al.*, 2008).

Approximately 15% of E/R-positive leukemias acquire an extra chromosome 21 as a secondary change (Attarbaschi *et al.*, 2004). Furthermore, near-tetraploid and near-triploid karyotype patterns in childhood B-cell precursor ALL were recently recognized as rather specific secondary abnormalities, although they occur in only approximately 5% of E/R-positive cases (Attarbaschi *et al.*, 2006; Raimondi *et al.*, 2006).

On the basis of these observations, we reasoned that E/R can affect chromosome segregation, most likely by compromising the surveillance mechanisms of the mitotic checkpoint (MC) (Weaver and Cleveland, 2005; Peters, 2007). The MC guarantees that all sister chromatids are properly attached to the spindles before cell-cycle progresses through mitosis. A central component of this checkpoint is the ‘mitotic arrest-deficient 2’ (MAD2) protein. Partial loss of function as well as over-expression of MAD2L1 leads to the abrogation of MC, chromosome mis-segregation, aneuploidy, and failure to arrest mitosis in the presence of microtubule poisons (Sotillo *et al.*, 2007). The unexpected finding of a tetraploid karyotype of Ba/F3 cells on stable expression of E/R prompted us to pursue this issue further. We have, therefore, investigated the influence of E/R on MC and especially on its major component MAD2L1.

Results and discussion

To assess the function of E/R, we used Ba/F3, a murine IL-3-dependent putative pro B-cell line, that is frequently used as a model cell line to study the effects of genes involved in the development of B-cell precursor ALL. For this purpose, we have created stable E/R-expressing Ba/F3 clones as reported earlier (Diakos *et al.*, 2007) and selected those with low expression of the chimeric protein. After continuous culture for approximately 2 months, all E/R-expressing clones ($n = 5$) acquired a tetraploid karyotype, whereas cells containing plasmids encoding either full-length ETV6, a truncated form of ETV6 (ETV6-F, with dominant-negative effect over ETV6 wt function) (Sasaki *et al.*, 2004), RUNX1, GFP, or an empty vector remained diploid. This change in ploidy was confirmed by DNA content

analysis and cytogenetics of several individual clones (Figures 1a and b). To corroborate the specificity of this effect, E/R-positive Ba/F3 clones and controls were synchronized by a short exposure to low doses of nocodazole and accumulated—unlike control cells—at 8N on a second exposure to the spindle toxin (Figure 1c). These findings indicate that tetraploidization is a specific effect of E/R-expressing Ba/F3 cells in long-term cultures.

Hence, we used diploid E/R-expressing Ba/F3, and several E/R-positive and E/R-negative leukemia cell lines to investigate the influence of the chimeric E/R protein on MC. Consistent with the notion that cells with an attenuated MC are unable to fully arrest their cell cycle in mitosis when treated with spindle poisons (Kops *et al.*, 2005), the proportion of E/R-positive cells with a 2N and 8N DNA content was significantly increased under the influence of nocodazole as compared with controls (Figures 2a and b; Supplementary Figures 1a and b). The higher percentage of E/R-positive cells with 8N implies that these cells did not accurately segregate their chromosomes and/or terminate cytokinesis. Determination of mitotic cells with the phospho-histone H3 staining, which is tightly correlated with chromosome condensation during mitosis, revealed consistently lower mitotic indexes in nocodazole-treated E/R-expressing Ba/F3 cell lines and leukemias, but not in E/R-negative model and leukemia cell lines (Figure 2c; Supplementary Figure 1c).

Given that the cell-cycle distribution assessed by DNA content analysis of E/R-positive Ba/F3 cell lines showed only a minor—statistically not significant—G0/1 increase compared with several controls (empty vector, expression vectors for RUNX1, ETV6, ETV6-F, or GFP) (Figure 2d), its deregulation is unlikely to be the cause of the low mitotic index, especially as cells were also measured after a long exposure to nocodazole. These findings accord with the observed reduction of G1 phase cells by RUNX1 and its increase by CBF oncoproteins (Lou *et al.*, 2000; Strom *et al.*, 2000; Ford *et al.*, 2009).

Of note and in line with a latent MC destabilization (Kops *et al.*, 2005), the spontaneous apoptosis rate of both E/R-positive Ba/F3 as well as E/R-positive leukemic cell lines were already slightly, but not significantly, higher than those of the respective parental Ba/F3 and E/R-negative control cell lines under optimal culture conditions (Figure 2e; Supplementary Figure 1d). We also excluded the possibility that nocodazole *per se* might selectively increase the apoptotic rate of mitotic E/R-positive cells by showing that the proportion of PI-positive cells did not increase considerably over basic levels (Figure 2f; Supplementary Figure 1e). The E/R-dependent MC impairment was further confirmed by the reduced levels of the checkpoint-associated protein securin, whose degradation at the start of anaphase initiates sister chromatid separation (Pines, 2006), and thus concords with the lower mitotic index (Supplementary Figure 1f). These findings strikingly resemble those recently obtained in similar experiments using acute myeloid leukemia samples and cell lines with a truncated RUNX1/ETO, a fusion protein that is closely related to E/R (Boyapati *et al.*, 2007; Wolyniec *et al.*, 2009).

Having shown the influence of E/R on MC function, we set out to unravel its potential molecular mechanism. For this purpose, we interrogated publicly available Affymetrix data sets from childhood ALL (Ross *et al.*, 2003) for the expression of genes implicated in MC function. *MAD2L1* and *BUB3* (budding uninhibited by benzimidazole 3), two key players of

MC, were consistently repressed in E/R-positive leukemias compared with all other ALL subgroups (Figure 3a). The finding that the MAD2L1 promoter region contained four perfect matches for RUNX binding (TGT/CGGT) and 13 sites with 85% homology within 3 kb upstream of the transcription start site makes it a likely candidate for a direct target of RUNX1 and also E/R. In contrast, no perfect RUNX-binding site was present within 3 kb of the promoter region of BUB3.

We, therefore, focused our further work on MAD2L1 and validated the expression of *MAD2L1* by quantitative RT–PCR in primary childhood ALL samples and confirmed a significant difference between E/R-positive and E/R-negative leukemias (Figure 3b). In accordance with mRNA expression, protein expression of *MAD2L1* was less abundant in E/R-positive leukemic and model cell lines (Figures 3c and d). Conversely, *MAD2L1* mRNA expression was up-regulated after E/R silencing in AT-2 and REH leukemic cell lines, further emphasizing its regulation by E/R (our unpublished observation).

To test the possibility that *MAD2L1* expression is directly regulated, we determined its transcriptional activity in the context of RUNX1 and E/R. Two constructs that contained endogenous promoter sequences with one or three perfect RUNX1 consensus sites were used for luciferase-based reporter assays (Guardavaccaro *et al.*, 2008). Both promoter constructs revealed a dose-dependent activation on RUNX1 transfection and E/R-associated repression of RUNX1-induced MAD2L1 activity in NIH3T3 cells (Figures 4a and b). Similarly, forced expression of RUNX1 led to induction of MAD2L1 promoter activation and coexpression of E/R to its abrogation in HEK293 cells (data not shown). In contrast, transfection of NIH3T3 cells with an expression vector with a point mutation in the runt domain (E/R_{R201Q}) that had been earlier shown to diminish DNA binding, but not heterodimerization with CBFβ (Song *et al.*, 1999; Li *et al.*, 2003), resulted in only partial reduction of RUNX1-induced activation (Figure 4c). These data accord with earlier studies and indicate that DNA binding is essential for E/R activity and that E/R modulates RUNX1-induced gene regulation (Hiebert *et al.*, 1996; Friedman, 1999; Morrow *et al.*, 2007; Wotton *et al.*, 2008; Roudaia *et al.*, 2009).

We then went on to show a direct *in vivo* interaction between E/R and the MAD2L1 promoter by ChIP analysis. Using Myc-RUNX1 or Myc-E/R stably expressing HEK293 cells, we were able to prove that both RUNX1 and E/R bind to the chromatin at two consensus RUNX1 sites, which were also present in the constructs used for the reporter assays (Figures 4d and e).

Collectively, these data suggest that direct suppression of MAD2L1 by E/R binding specifically adds to the destabilization of the MC (Perez de Castro *et al.*, 2007). However, as only a small subgroup of E/R-positive leukemias acquires non-random-specific numerical chromosomal abnormalities (Attarbaschi *et al.*, 2004, 2006; Raimondi *et al.*, 2006), it seems unlikely that MAD2L1-associated MC attenuation alone is responsible for this phenomenon. Furthermore, it remains to be investigated whether the E/R-induced deregulation of MAD2L1 or any other MC components might also contribute in other ways to leukemogenesis, such as perhaps p53 activation or deregulation of the DNA damage

response pathways (Michel *et al.*, 2004; Kops *et al.*, 2005; Fang *et al.*, 2006; Ha *et al.*, 2007; Perez de Castro *et al.*, 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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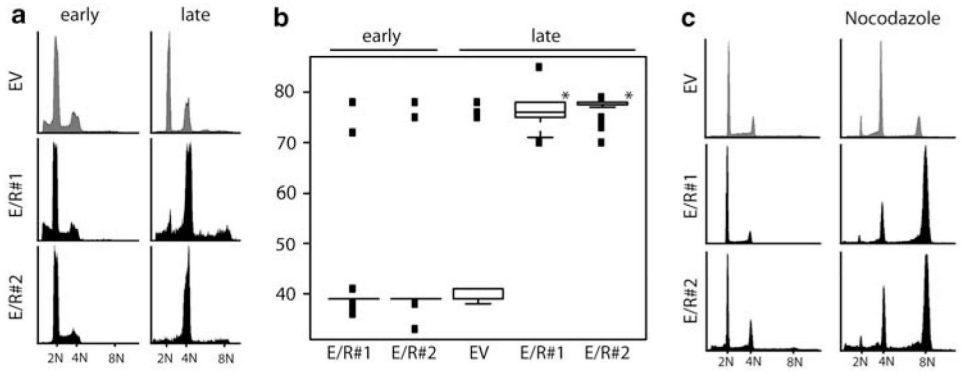

Figure 1.

E/R expression induces a tetraploid karyotype in Ba/F3 cells. Representative histograms visualizing DNA content analysis (PI) of two E/R + clones (#1 and #2) and empty vector (EV) control clones at early and late passages. (**b**) Box blot analysis depicting chromosome numbers of E/R-expressing or EV Ba/F3 cells from cultures as in (**a**). Welch's *t*-test, **P*≤0,05. (**c**) DNA content analysis of untreated (left part) and synchronized E/R and EV Ba/F3 clones on nocodazole treatment (right part). Cells were released from the first arrest (nocodazole 75 ng/ml for 14 h) for 3 days before they again were exposed to nocodazole. One of at least three independent experiments is shown.

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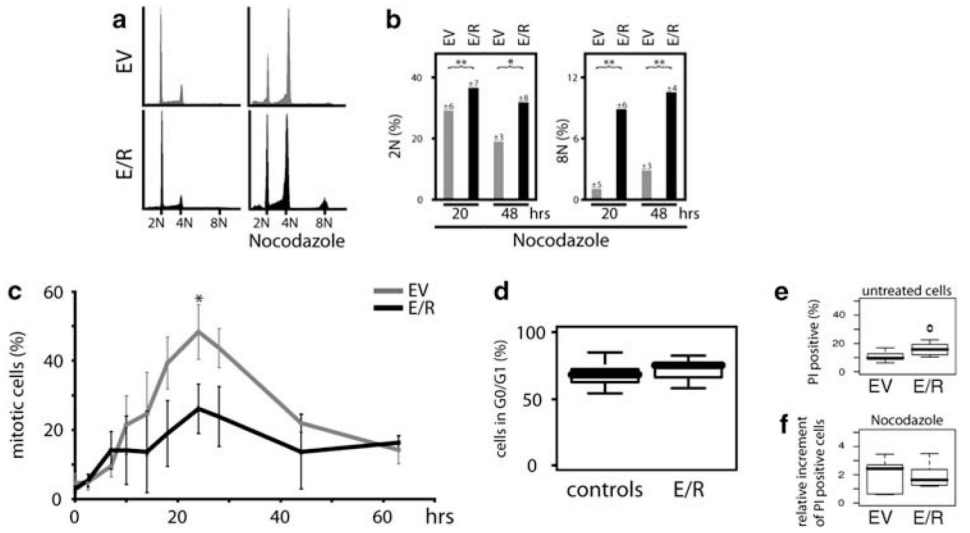

Figure 2.

E/R attenuates the MC. Cell-cycle distribution of a mixture of three Ba/F3 clones that stably expressed E/R or contained an empty vector (EV) by DNA content analysis. (**a**) Shown are representative histograms in the absence (left) and presence (right) of nocodazole (333 ng/ml for 48 h). (**b**) Bar graphs indicate percentages of E/R-expressing and EV Ba/F3 cells with 2N and 8N after treatment with nocodazole for 20 and 48 h. The median and standard deviation (indicated at the top of each bar) are taken from five different experiments. Welch's *t*-test, **P*≤0.05; ***P*≤0.01. (**c**) Mitotic index of stably E/R-expressing and EV Ba/F3 cells was determined by phospho-histone H3 staining on MC activation by nocodazole for the indicated times. Experiments depict the average and standard errors of the mean of three individual experiments. Welch's *t*-test, **P*≤0.05. (**d**) Cell-cycle distribution of Ba/F3 clones stably expressing E/R or various control vectors (ETV6, truncated ETV6, RUNX1, GFP, or EV) by DNA content analysis with PI. Box blots depict the percentage of cells in G0/G1 of E/R + clones and controls from 5 and 7 independent experiments, respectively. (**e/f**) Apoptosis rates were assessed by PI staining in stably E/R-expressing and EV Ba/F3 cells. Percentages of PI-positive cells are depicted under optimal culture conditions (**e**) and on nocodazole treatment for 24 h relative to untreated cells (**f**). Welch's *t*-test, $P = 0.8$.

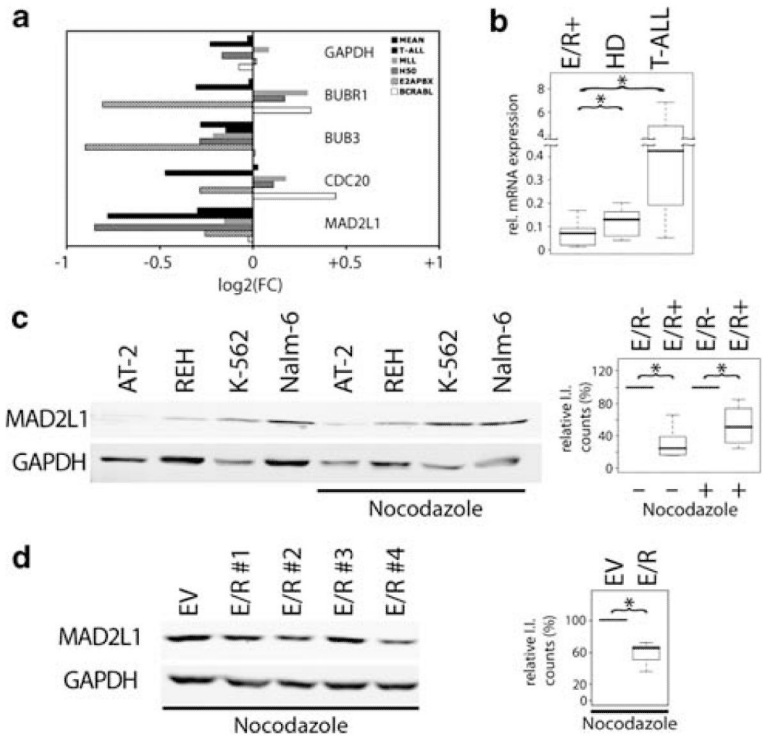

Figure 3.

E/R represses MAD2L1 expression. (**a**) Micro-array analysis of MAD2L1 expression in various subgroups of childhood ALL. Published data were analyzed for M-values (log2(FC)) of probe sets from genes instrumental in MC function. Shown is the *MAD2L*1 expression from E/R-positive leukemias relative to their E/R-negative counterparts in each leukemic subgroup. Mean, the mean of all leukemic groups; T-ALL, T-ALL; MLL, ALL with MLL fusion genes; H50, high hyperdiploid ALL; E2APBX, TCF3/PBX1 (E2A/PBX1) gene rearrangement positive ALL; BCRABL, ALL with BCR/ABL fusion gene. (**b**) MAD2L1 mRNA was quantified by TaqMan qRQ-PCR and the ABI Prism 7900 detection system (Applied Biosystems, Foster City, CA, USA) in 10 cases each of the after distinct childhood ALL subgroups: E/R +, high hyperdiploid (HD) and T-cell precursor (T-ALL). The *ABL* gene was used as a standard reference for normalization. Box plots display the relative expression of *MAD2L1* using STS 2.2 software. Shown are the median and interquartile range, 95% of the values are within the range of a whisker. Median values were used for statistical analysis; Welch's *t*-test, **P*≤0.05. (**c**) Western blot analysis was performed from whole cell lysates of E/R-positive (REH, AT-2) and E/R-negative leukemic cell lines (K562 and Nalm-6). The MAD2L1 protein was detected with a specific anti-MAD2L1 antibody (ab 3632, Abcam, Cambridge, UK). Anti-GAPDH (6C5, Santa Cruz Biotechnology, CA, USA) was used as loading control. Left: protein levels under standard culture conditions and on nocodazole treatment (50 ng/ml nocodazole, 18 h). Right: box plots show the quantification of MAD2L1 protein in the respective groups from four independent experiments. MAD2L1 protein was normalized to GAPDH expression using the LICOR software (I.I., Integrated Intensity counts). Welch's *t*-test, **P*≤0.05. (**d**) Western

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blot analysis (left) and quantification (right) of MAD2L1 in E/R + Ba/F3 clones and EV controls. One of three independent experiments is shown. Welch's *t*-test, $*P \leq 0.05$.

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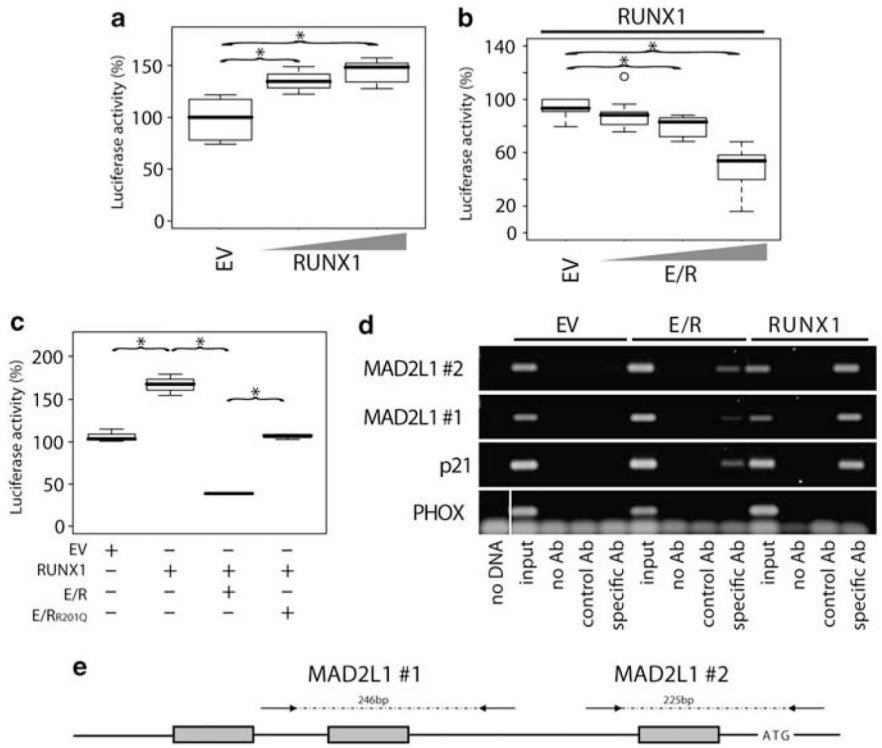

Figure 4.

Direct regulation of MAD2L1 by RUNX1 and abrogation of this effect by E/R. Luciferase assays were performed using a 1.2 kb MAD2L1 genomic reporter fragment containing three perfect RUNX1-binding sites (**a**–**c**). NIH3T3 cells were transfected (JetPEI Polyplus transfections, Illkirch, France) with RUNX1, E/R, E/R_{R201Q}, or a combination thereof. Cells were cotransfected with CBFβ (12.5 ng) to enhance RUNX1 binding and TK Renilla for transfection control. The total amount of DNA for each experiment was kept constant. Values obtained from EV control samples were set to 100%. Luciferase activity is depicted using (**a**) increasing amounts of RUNX1 pcDNA3.1-expression vector (100 and 500 ng) or (**b**) E/R (200, 400, and 800 ng) in addition to RUNX1 (400 ng) to show dose-dependent reporter activation by RUNX1 and its repression by E/R, respectively. Box plots of three representative experiments are depicted. Welch's *t*-test, **P*≤0.05. (**c**) NIH3T3 cells were transfected using various combinations of 267 ng empty vector (EV), E/R, or a DNA-binding mutant E/$R_{R201Q,}$ and/or 100 ng RUNX1-expression vector. One of three representative experiments is depicted. Welch's *t*-test, **P*≤0.05. (**d**) RUNX1 and E/R occupation of MAD2L1 promoter region *in vivo* was assessed by ChIP. Chromatin from Myc-tagged E/R, Myc-RUNX1, or Myc-tagged EV stably expressing HEK293 cells was immunoprecipitated with anti-c-Myc (9E11, Abcam) or control antibody (anti-N cadherin, 610920, BD). Aliquots of the isolated DNA fragments (without antibody (noAb), with control or specific Ab and input DNA) were subjected to PCR with specific primers that amplify regions of the MAD2L1 promoter (Supplementary Methods) and analyzed by DNA gel electrophoresis. Specific primers were used to amplify specific regions of $p21^{WAF1}$ and PHOX promoters as positive and negative controls, respectively. The gel from one of two independent experiments is shown. A vertical line has been inserted to indicate where the

gel lane was cut. These gels came from the same experiment. (**e**) Schematic diagram of the 1.2 kb of the promoter region of the MAD2L1 gene cloned into the pGL3. The three perfect RUNX1-binding motifs (TGT/CGGT) are indicated by gray boxes. Arrows flanking the two RUNX1 sites (#1 and #2) indicate primer positions used for PCR amplification of isolated DNA fragments after ChIP. Numbers refer to the size of the expected PCR product. Graph is not to scale.

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