Molecules and Cells

Involvement of RUNX and BRD Family Members in Restriction Point

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A tumor is an abnormal mass of tissue that arises when cells divide more than they should or do not die when they should. The cellular decision regarding whether to undergo division or death is made at the restriction (R)-point. Consistent with this, an increasingly large body of evidence indicates that deregulation of the R-point decision-making machinery accompanies the formation of most tumors. Although the R-point decision is literally a matter of life and death for the cell, and thus critical for the health of the organism, it remains unclear how a cell chooses its own fate. Recent work demonstrated that the R-point constitutes a novel oncogene surveillance mechanism operated by R-point–associated complexes of which RUNX3 and BRD2 are the core factors (Rpa-RX3 complexes). Here, we show that not only RUNX3 and BRD2, but also other members of the RUNX and BRD families (RUNX1, RUNX2, BRD3, and BRD4), are involved in R-point regulation.

Keywords: BRD, restriction point, RUNX

INTRODUCTION

Cells consult their extracellular environment and growth-regulating signals during a discrete window of time in the G_1 phase of the cell cycle. If growth factors are removed before cells decide whether to divide, the cells will not proceed further into the cell cycle, and instead enter G_0 phase. However, once cells have moved through the decision-making period, removal of serum no longer affects their progress, and they proceed through S, G_2, and M phases (Pardee, 1974). This schedule, in which the cell is totally dependent on extracellular signals early in G_1, but then becomes independent of these signals, implies that an important decision must be made before the end of early G_1. Precisely at this point, a cell must 'make up its mind' whether it will remain in G_1, retreat from the active cycle into G_0, or advance into late G_1 and proceed with the remaining phases of the cell cycle. This critical decision point is called the restriction point or R-point (Malumbres and Barbacid, 2001; Pardee, 1974; Weinberg, 2007). In most mammalian cells studied to date, the R-point occurs 3 to 4 h after mitogenic stimulation and several hours before the G_1/S-phase transition (Zetterberg et al., 1995). Deregulation of the R-point is strongly associated with deregulation of proliferation and apoptosis in most tumor cells (Pardee, 1974; Weinberg, 2007). Therefore, a better understanding of how cells choose their own fate at the R-point will provide insight into how tumors develop.

When extracellular mitogenic signaling is maintained up to the R-point, transcriptional activation of R-point–associated target genes is activated (Chi et al., 2017). For silent genes to be induced, target-binding sites within their regulatory promoter regions must be bound *de novo* by transcription factors, which initiate gene expression. The special transcription factors that have the function to associate with condensed chromatin independently of other factors and modulate

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chromatin accessibility are noted as pioneer factors (Zaret and Carroll, 2011). We recently showed that acetylated RUNX3 interacts with BRD2 and functions as a pioneer factor of the R-point (Lee et al., 2019). BRD2, which contains two bromodomains recognizing acetylated lysine residues (Huang et al., 2007; LeRoy et al., 2008), bridges acetylated RUNX3 (an enhancer binding protein) with acetylated histones and recruits the basal transcription machinery, histone-modifying complexes, and chromatin-remodeling complexes that enable R-point–associated target gene expression (Lee et al., 2019). This complex, of which RUNX3 and BRD2 are the core components, was named the R-point–associated RUNX3 containing activator complex (Rpa-RX3-AC) (Lee et al., 2019). After the R-point decision is made, Rpa-RX3-AC interacts with the PRC1-HDAC4-Cyclin D1 complex to form the R-point–associated RUNX3-containing transient complex (Rpa-RX3-TR). Soon thereafter, when the oncogenic signal is downregulated by an inhibitory feedback loop, RUNX3 dissociates from BRD2 to form the R-point–associated RUNX3-containing repressor complex (Rpa-RX3-RE). The cell then passes through the R-point, and the cell cycle progresses toward S-phase. When K-RAS is constitutively activated, the Rpa-RX3-AC is maintained for a long period of time, leading to a death decision (Lee et al., 2019). Therefore, the Rpa-RX3-AC→Rpa-RX3-TR→Rpa-RX3-RE transition constitutes a unique oncogene surveillance mechanism for sensing duration of the mitogenic signal.

Mammalian species have three RUNX family genes (*RUNX1*, *RUNX2*, and *RUNX3*) that share the highly conserved Runt-domain (Fig. 1A). All three RUNX genes exhibit distinct tissue-specific expression patterns and are intimately involved in carcinogenesis: *RUNX1* mutation has been extensively studied in human leukemia (Mangan and Speck, 2011); *RUNX2* encodes a bone lineage-specific factor that is linked to osteosarcoma (Pratap et al., 2006); and inactivation of *RUNX3* in solid tumors plays a causative role in cancer pathogenesis (Ito, 2008; Ito et al., 2015). In addition, not only BRD2, but also other BRD family members (BRD3, BRD4, and BRDT) contain two highly conserved bromodomains that recognize acetylated lysine residues (Filippakopoulos et al., 2012) and are mutated in various tumors (Belkina and Denis, 2012). Hence, we investigated whether members of RUNX and BRD families, in addition to RUNX3 and BRD2, are involved in R-point regulation.

MATERIALS AND METHODS

Cell lines

HEK293 cells were maintained in Dulbecco's modi ed Eagle's medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin (Invitrogen, USA).

DNA transfection, immunoprecipitation (IP), and immunoblotting (IB)

Transient transfections in all cell lines were performed using Lipofectamine Plus reagent and Lipofectamine (Invitrogen). Cell lysates were incubated with the appropriate monoclonal antibodies for 3 h at 4°C, and then with protein G–Sepharose beads (Amersham Pharmacia Biotech, USA) for 1 h at 4°C. For detection of endogenous proteins, lysates were incubated with the appropriate monoclonal antibodies for 6 to 12 h at 4°C, and then with protein G–Sepharose beads (Amersham Pharmacia Biotech) for 3 h at 4°C. Immunoprecipitates were resolved on SDS-PAGE gels and transferred to a polyvinylidene difluoride membrane (Millipore, USA). The membrane was immunoblotted with the appropriate antibodies after blocking and visualized on an Amersham™ Imager 600 (GE Healthcare, USA) after treatment with ECL solution (Amersham Pharmacia Biotech).

Antibodies

Antibodies targeting RUNX3 (5G4) and BRD4 (ab128874) were obtained from Abcam (UK). Antibodies targeting FLAG (M2; Sigma, USA), Myc (9E10; Santa Cruz Biotechnology, USA), BRD2 (M01; Abnova, Taiwan), and BRD3 (sc-81202; Santa Cruz Biotechnology) were used for IP and IB.

RESULTS AND DISCUSSION

Previously, we showed that the first bromodomain (BD1) of BRD2 specifically recognizes acetylated lysines 94 and 171 (Ac-K94 and Ac-K171) of RUNX3 (Lee et al., 2013). These lysine residues and surrounding amino acid sequences are highly conserved among RUNX family members (RUNXs) (Fig. 1A). To determine whether RUNX1 and RUNX2 also interact with BD1 of BRD2, we generated FLAG-tagged-BRD2 (FLAG-BRD2) in which tyrosine-113 or tyrosine-386 was replaced by phenylalanine (Y > F) within BD1 (FLAG-BRD2-M1) or BD2 (FLAG-BRD2-M2), respectively (Fig. 1B). Each mutation abrogates the interaction of the corresponding BD with acetylated lysines (Cheung et al., 2017). We then co-transfected Myc-tagged-RUNX1 (Myc-RUNX1) and FLAG-tagged wild-type BRD2 (FLAG-BRD2-WT) or mutated BRD2 into HEK293 cells, and analyzed the interaction between RUNX1 and BRD2 by co-IP followed by IB. RUNX1 interacted with BRD2-WT and BRD2-M2, but not with BRD2-M1 (Fig. 1C). Similarly, RUNX2 interacted with BRD2-WT and BRD2-M2, but not with BRD2-M1 (Fig. 1D). Thus, RUNX1 and RUNX2, as well as RUNX3, interact with BRD2, and these interactions are mediated through the same domain of BRD2, i.e., BD1.

The RUNX3–BRD2 interaction occurs 1 h after serum stimulation, and is maintained for up to 2 h afterward. During that time, the R-point decision is made, and then the RUNX3–BRD2 complex dissociates (Lee et al., 2019). To determine whether other RUNX and BRD family members are also involved in the R-point decision-making machinery, we expressed Myc-RUNX1, Myc-RUNX2, or Myc-RUNX3 in HEK293 cells and measured the timing of the interaction between the exogenously expressed RUNXs and endogenous BRDs by co-IP and IB. All RUNX family members formed complexes not only with BRD2, but also with BRD3 and BRD4, 2 h after serum stimulation, and the complexes dissociated thereafter (Fig. 1E). Importantly, when the constitutively active form of K-RAS (K-RAS^{G12V}) was expressed, all complexes were formed even in the absence of serum stimulation and maintained for a long time after serum stimulation (Fig. 1F). These results indicate that not only RUNX3 and BRD2, but also other RUNXs

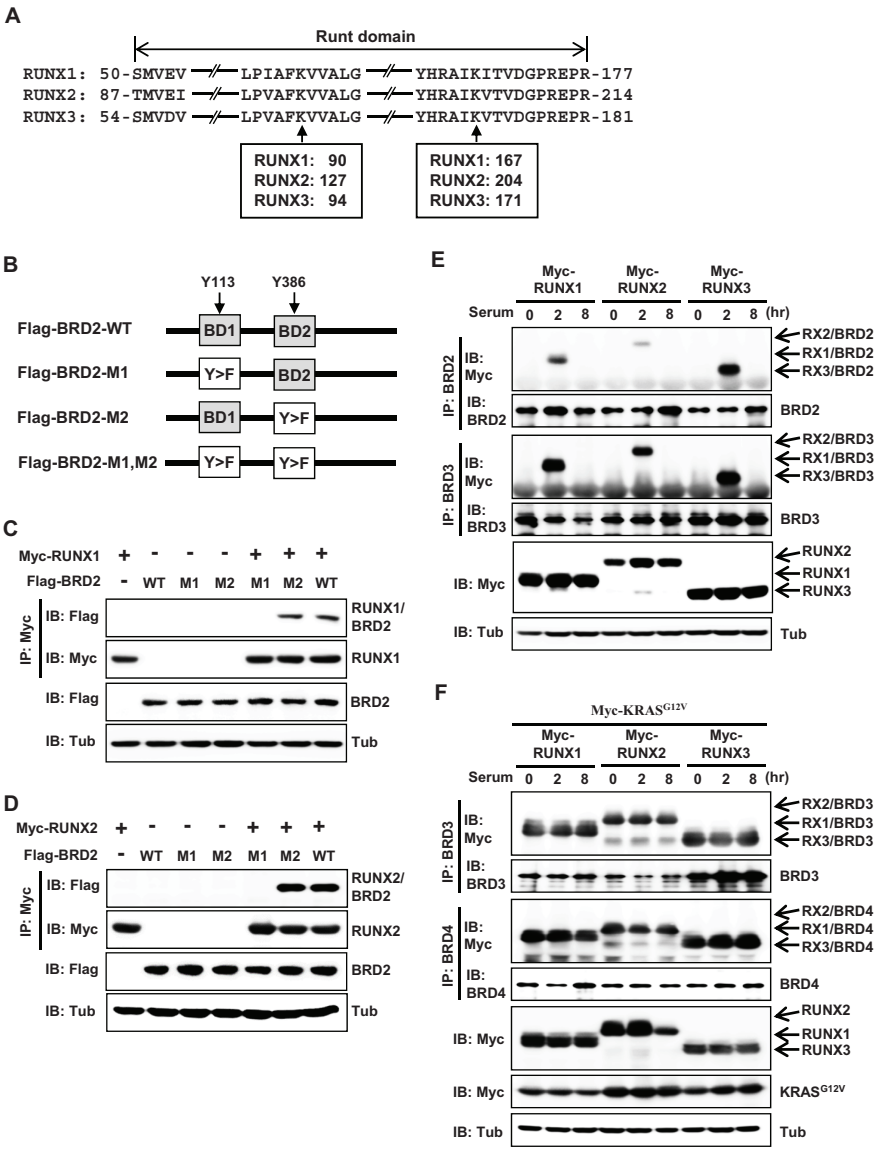

Fig. 1. Transient interaction between RUNXs and BRDs early after serum stimulation. (A) Amino acid sequence similarities between RUNX1, RUNX2, and RUNX3. Conserved lysines (K) recognized by bromodomain 1 (BD1) of BRD2 are indicated. (B) Schematic of wild-type (BRD2-WT) and mutant BRD2. BD1 interacts with RUNX3 acetylated at K-94 and K-171; BD2 interacts with acetylated histones H4K4-ac, H4K12-ac, and H3K14-ac. Y113 and Y386 are essential tyrosines in BD1 and BD2, respectively. Y > F indicates a tyrosine-to-phenylalanine mutation. (C) HEK293 cells were transfected with Myc-RUNX1, FLAG-BRD2-WT, and FLAG-BRD2 mutant described in Figure 1B. Cells were serum-starved for 24 h, and then stimulated with 10% serum. Cells were harvested 2 h later, and the interactions of the proteins were measured by co-IP and IB, as indicated. (D) HEK293 cells were transfected with Myc-RUNX2, FLAG-BRD2-WT, and FLAG-BRD2 mutant, and then treated as described in Figure 1C. Protein-protein interactions were measured by IP and IB as indicated. (E) HEK293 cells were transfected with Myc-RUNX1, Myc-RUNX2, or Myc-RUNX3; serum-starved for 24 h; and then stimulated with 10% serum. Cells were harvested at the indicated time points, and the levels of the indicated proteins and their time-dependent interactions were measured by co-IP and IB. (F) HEK293 cells were transfected with Myc-KRASG12D with Myc-RUNX1, Myc-RUNX2, or Myc-RUNX3, and treated as described in Figure 1E. The levels of the indicated proteins and their time-dependent interactions were measured by co-IP and IB.

and BRDs, are involved in the R-point decision-making machinery and contribute to oncogene surveillance.

BRD2, BRD3, and BRD4 are ubiquitously expressed, whereas BRDT is expressed specifically in the testis (Taniguchi, 2016). By contrast, RUNX family members are expressed in a tissue-specific manner (Ito et al., 2015). Our results suggest that each RUNX family member, along with the BRD proteins, contributes to R-point regulation in the tissue in which it is expressed. The RUNX3–BRD2 interaction initiates formation of Rpa-RX3-AC, which transitions to Rpa-RX3-TR. After the R-point, RUNX3 dissociates from BRD2 and forms Rpa-RX3-RE. Therefore, RUNX1 and RUNX2 may also sequentially form

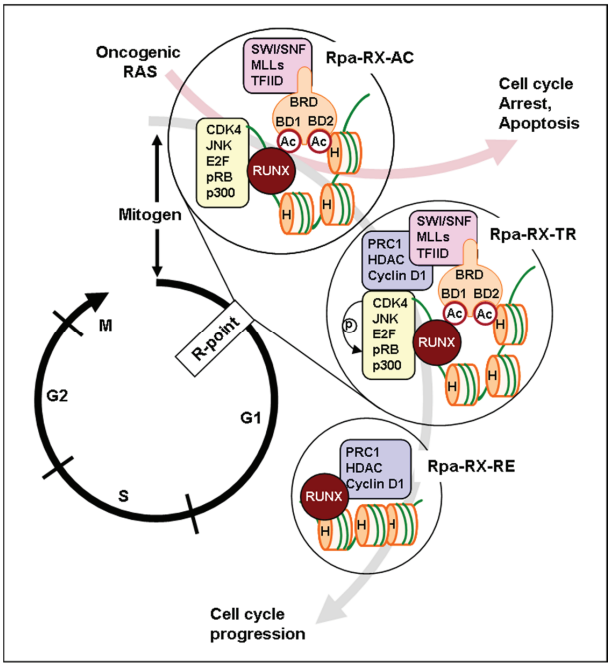

Fig. 2. The predicted sequential molecular events for the R-point decision. Upon mitogenic stimulation, p300 associates with RUNXs and acetylates RUNXs and histones. One hour after mitogenic stimulation, BRDs bind both acetylated RUNXs and acetylated histone through their bromodomains. Subsequently, SWI/SNF, MLL1/5, and TFIID bind to the C-terminal region of BRDs. The large complex of which RUNXs are the core was named Rpa-RX-AC. When the RAS-MEK signal is downregulated, the Cyclin D1-HDAC4-PRC1 complex binds to Rpa-RX-AC, yielding Rpa-RX-TR. The Cyclin D1-HDAC4-PRC1 complex dissociates from Rpa-RX-TR to form Rpa-RX-RE. The cell cycle then progresses toward S-phase. If K-RAS is constitutively activated, the RAS-MEK signal is not downregulated, and Rpa-RX-AC is maintained for a long time; consequently, the cell cycle arrests or the cell undergoes apoptosis.

Rpa-RX-AC, Rpa-RX-TR, and Rpa-RX-RE in order to regulate R-point regulation (Fig. 2).

In summary, we have shown that not only RUNX3, but also RUNX1 and RUNX2, which play critical roles in determination of the hematopoietic and osteogenic lineages, respectively, are involved in R-point regulation, implying that all RUNXs contribute to lineage-specific R-point decision-making. Future studies should seek to identify the mechanism by which RUNXs and BRDs collaborate to make these lineage-specific decisions.

Disclosure

The authors have no potential conflicts of interest to disclose.

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