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Expression of Ligands for the NKG2D Activating Receptor are Linked to Proliferative Signals

by Heiyoun Jung

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Professor David H. Raulet, Chair
Professor Mark S. Schlissel
Professor Stuart Linn
Professor Gertrude Buehring

ABSTRACT

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NKG2D is a stimulatory receptor expressed by natural killer cells and subsets of T cells. The receptor recognizes a set of self-encoded cell surface proteins that are usually not displayed on the surface of healthy cells but are often induced in transformed and infected cells. NKG2D engagement activates or enhances the cell killing function and cytokine production programs of NK cells and certain T cells. Emerging evidence suggests that different ligands are to some extent regulated by distinct signals associated with disease states, thus enabling the immune system to respond to a broad range of disease-associated stimuli via a single activating receptor.

The research presented in this thesis demonstrated that at least one of the murine NKG2D ligands, RAE-1 ε (gene: *Raet1e*), is transcriptionally activated by signals associated with cell proliferation. Primary cultured fibroblasts from normal tissue, which did not express RAE1 *in vivo*, were induced to express large amounts of cell surface RAE-1 ε upon culture *in vitro*. RAE-1 ε induction was associated with increased *Raet1e* transcription. Inhibitor and other experiments showed that RAE-1 ε induction was dependent on sustained cell proliferation induced by growth factors, but was not dependent on a variety of other pathways, including the DNA damage response, oxidative stress or serum components other than growth factors. *In vivo*, correlative, evidence showed that RAE-1 ε was also displayed on rapidly proliferating brain cells in early embryos, but was extinguished at later stages of brain development as cell proliferation slowed.

In line with these findings, analysis showed that the *Raet1e* promoter was more active in proliferating cells than quiescent cells. *In silico* analysis of the *Raet1e* promoter for potential binding sites for transcription factors associated with cell cycle regulation revealed multiple putative binding sites for E2F family members. Chromatin immunoprecipitation studies demonstrated that the *Raet1e* promoter was bound *in vivo* by E2F family members in proliferating cells. Overexpression of activating E2F family

members induced endogenous *Raet1e* transcripts in nonproliferating cells and transactivated a *Raet1e* promoter reporter plasmid. Transactivation was strongly inhibited if two putative E2F sites in the promoter were mutated.

Collectively, the data show that transcription factors that regulate cell proliferation regulate the transcriptional activation of cell surface ligands that target transformed and infected cells for destruction by NK cells and T cells. E2F family members are often mutated in cancer and proliferative signals often accompany viral infections. On the other hand, many healthy cells undergo proliferation without induction of RAE-1 on the cell surface, so it appears that proliferation by itself is not always sufficient to induce RAE-1 expression at the cell surface. Other stress pathways activated during tumorigenesis or in infected cells are likely to work together with the proliferative signal documented here to ensure that cell surface expression of NKG2D ligands is restricted to unhealthy cells.

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Heiyoun Jung
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Chapter1. Introduction

Introduction to the mammalian immune system

The immune system is classified into two major arms: the innate immune system and the adaptive immune system. The innate immune system serves as a first protective barrier to pathogens or 'malfunctioning' self. A variety of cell types mediate the innate immune response, including dendritic cells, macrophages, granulocytes and NK cells. NKT cells, γδ T cells are also sometimes considered to be innate immune cells, despite their expression of T cell antigen receptors. Innate immune cells typically respond very rapidly after an infection or other threat is encountered.

One function of innate immune cells is to recognize various pathogen associated molecular patterns (PAMPs) to directly sense pathogens or infected cells. In addition, some cells, such as NK cells and γδ T cells, can recognize tumor cells or diseased cells, even though the latter cells do not necessarily express foreign antigens. The various innate cells play a crucial role in shaping and enhancing adaptive immunity. The cells of the adaptive immune system, B and T cells, take longer to respond to a threat, but produce immune responses with a higher degree of specificity that is often more potent. Furthermore, the adaptive immune system generates memory cells that can respond very rapidly to a second exposure to a pathogen or tumor in an antigen-specific manner.

Natural killer cells

Natural killer (NK) cells are a subset of lymphocytes that develop in the bone marrow from a common lymphoid progenitor cell (CLP) (Kondo, et al., 1997). Unlike T cells and B cells, which also arise from CLP, NK cells each express several specific stimulatory and inhibitory receptors, and the receptors are germline-encoded as opposed to somatically diversified. Also, NK cells are functionally distinct from B and T cells due to their ability to kill target cells without priming. Due to this ability, NK cells are involved in a variety of innate immune response against viruses, bacteria and other pathological alterations, such as malignant transformation. Furthermore, NK cells regulate adaptive immune responses by stimulating Dendritic cells, and possibly T cells (Raulet, 2004).

NK cell receptors

With a variety of receptors, mature NK cells are capable of distinguishing normal cells from cells that are stressed as a result of infection or transformation. The receptors fall into two major classes distinguished by their ability to induce stimulatory vs. inhibitory signaling cascades. The balance between inhibitory and stimulatory signals decides the response of an individual NK cell. When stimulatory signals outweigh the inhibitory ones over some threshold, NK cells respond (Raulet, 2004).

Inhibitory receptors

An important group of receptors expressed by mouse and human NK cells recognizes MHC class I molecules, and therefore inhibits lysis of cells with high MHC I expression and conversely allows lysis of tumor cells or infected cells that have downregulated MHC I (missing self recognition).

Inhibitory function is primarily mediated by the Ly49 family of receptors in mice and the KIR family in human, which recognize MHC class I. Also, the CD94/NKG2A heterodimer recognizes the non-classical MHC I molecule, Qa-1b in mice and HLA-E in human, associated with peptides derived from classical MHC class I molecule. All of these receptors contain an immunoreceptor tyrosine-based inhibitory (ITIM) motif that recruits phosphatases that de-phophorylate important signaling intermediates, and therefore results in dampening of the NK response. Notably, in addition to their impact on effector cell functions, signals received through these receptors under steady state conditions play a major role in establishing the responsiveness threshold of NK cells (Joncker, et al. 2010). This mechanism ensures that the few NK cells that arise in normal animals that lack inhibitory receptors remain self-tolerant.

In addition, NK cells express inhibitory receptors specific for non-MHC ligands, whose roles are less well understood. These include NKRP1B and NKRP1D, which recognize Clr-b, and KLRG-1, which recognizes cadherin.

Stimulatory receptors

One of the best-studied stimulatory receptors on NK cells is NKG2D, which will be discussed in detail later in this introduction. A second group of stimulatory receptors, called natural cytotoxicity receptors (NCR), include NKp30, NKp44, NKp46 and NKp80. Reported ligands for NCRs includes viral hemagglutinins for NKp44 and NKp46 and B7-H6 for NKp30. Furthermore, antibody specific for NCRs can block NK cell killing of certain tumor cells, which suggests that the corresponding ligands are expressed on these tumor cells.

A few members of Ly49 family encode stimulatory receptors. One of well-characterized example is Ly49H, which recognizes the viral (MCMV) glycoprotein m157 that resembles MHC class I. A subset of human KIRs are stimulatory receptors as well. The stimulatory KIR and Ly49 receptors lack ITIMs in their cytoplasmic domain, and instead associate noncovalently with a signaling adapter molecule called DAP12, which contains ITAMs.

DNAM-1 is another stimulatory receptor expressed by NK cells, and is also expressed by T cells and macrophages. DNAM-1 recognizes nectin-2 (CD112) and PVR (CD155).

Members of the SLAM receptor family also serve as stimulatory receptors for NK cells, although they can also impart inhibitory signals in certain conditions (Dong 2009). The SLAM family consists of SLAM, 2B4, Ly-9, CD84 and CRACC, all of which are triggered by homotypic receptor-ligand interaction except 2B4, which recognizes CD48. The SLAM family is reported to play a role in recognition of

hematopoietic tumor cells or nontransformed hematopoietic cells lacking MHC I (Dong, et al., 2009). Another NK receptor in C57B1/6 mice, NKRP1C, is also a stimulatory receptor, but its ligands are not yet known. NKRP1C is the pan NK marker recognized by NK1.1-specific antibodies.

The NKG2D Receptor and its ligands

NKG2D is a homodimeric receptor that is expressed on all NK cells, as well as activated CD8 T cells, and subsets of γδ T cells, NKT cells and CD4 T cells. Despite its name, NKG2D shares little relatedness with other NKG2 members except that they are encoded near each other in the NK gene complex. NKG2D does not pair with CD94 or recognize HLA-E/Qa-1, as do NKG2A, -C and E. Instead, NKG2D recognizes a distinct family of self-ligands that are distantly related to MHC I molecules (described in the next section).

NKG2D provides activating signals via associated signaling adapter molecules, but the signaling adapters used vary in different conditions and in different species. In mice, two isoforms of NKG2D generated by alternative splicing differ at the N-terminus of the cytoplasmic domain and associate with different signaling adapter molecules (Diefenbach, et al., 2000). NKG2D in unstimulated mouse NK cells is primarily the shorter form, which pairs the adapter DAP10. But once NK cells are activated by polyI:C or IL-2, a longer form of NKG2D is also expressed, which pairs with both DAP12 and DAP10. Upon NKG2D engagement, the ITAM in DAP12 recruits ZAP70 or Syk tyrosine kinases. In contrast, DAP10 lacks an ITAM, and instead contains a YINM motif that closely resembles those found in co-stimulatory molecules. The YINM motif recruits PI3K and Grb2 upon activation. Activation through DAP10 and DAP12 provides sufficient signals to induce cytolysis and IFN-g production, so that NKG2D serves as a fully competent stimulatory receptor on NK cells. In contrast, in human NK cells and also in mouse and human CD8 T cells, NKG2D associates exclusively with DAP10. In CD8 T cells, a number of studies have demonstrated that NKG2D serves as a costimulatory receptor (Diefenbach, et al., 2002, Groh, et al., 2001, Jamieson, et al., 2002), although there is still controversy on this point (Ehrlich, et al., 2005).

NKG2D ligands

NKG2D ligands are diverse and all are distantly related to MHC class I. In mouse, In mice, the ligands include five members of the Rae1 (a-e) subfamily, Mult1 and three members of the H60 (a-c) family. In humans, the ligands include RAET1s (also known as ULBPs), MICA and MICB. They all share a similar N-terminal domain consisting of two Ig-like domains (a1, a2), but they differ in that MICA and MICB contain a3 Ig-like domains (which do not bind to b2m) whereas RAET1 proteins do not.

Most NKG2D ligands in both mouse and human are attached to the cell surface via GPI anchors, but a few of them are instead conventional type 1 membrane-spanning proteins (including H60a, Mult1 and MICA/MICB, RAET1G) of NKG2D to its ligands is greater than that of most immunoreceptor-ligand interactions (Raulet, 2003).

The diverse nature of ligands for one receptor suggests that they have evolved under selective pressure from pathogenesis. One of the best-documented examples suggesting this possibility is the evasion of NKG2D mediated recognition by human and mouse cytomegaloviruses. Infections with both viruses induce expression of NKG2D ligand mRNAs in infected cells, but both viruses also encode proteins that specifically inhibit expression of one or more NKG2D ligand at the protein level. Different subfamilies of ligands are inhibited by different viral evasins, suggesting an evolutionary back-and-forth between the virus and the host. Another possible explanation for the existence of multiple ligands is to provide a level of diversity that allows different ligands to be regulated by different cellular stress pathways, as suggested by recent reports (Gasser, et al., 2005, Nice, et al., 2009, Whang, et al., 2009). In this way, a single receptor (NKG2D), can be employed for responses to different types of stress stimuli.

Expression and regulation of NKGD ligands

NKG2D ligands are induced on stressed cells and are generally poorly expressed by normal cells. However, exprssion of NKG2D ligands by normal cells has been reported in some instances. In mice, *Raet1* mRNA is observed during embryogenesis from day 9-14 (Zou, et al., 1996), and it has been reported that BALB/c bone marrow cells can express significant levels of NKG2D ligands (Ogasawara, et al., 2005). In adult mice, a low level of RAE1 can reportedly be detected on hepatocytes (Vilarinho, et al., 2007), and H60c transcripts are abundantly expressed in skin tissue (Takada, et al., 2008) (Whang, et al., 2009). In human, ULBPs (RAET1) transcripts are detected in different healthy tissues, including kidney, prostate, uterus, tonsil and lymph nodes (Cosman, et al., 2001). The ULBP4 transcript is specifically abundantly detected in the skin (Chalupny, et al., 2003). Also, northern blot analysis of MICA and MICB expression in various healthy organs showed that both MICA and MICB are widely transcribed in many tissues, except the central nervous system (Schrambach, et al., 2007). However, whether these ligands are expressed on the cell surface has not been addressed, and it is likely that the expression is tightly regulated by post-transcriptional mechanisms to ensure NK cells are not activated against healthy normal tissues.

NKG2D ligands can be upregulated by multiple stimuli. Rae1 is expressed on the surface of the majority of immortalized mouse tumor cell lines and established cell lines derived from normal cells, and in many primary tumors (Diefenbach, et al., 2002), (Guerra, et al., 2008). MICA and MICB are expressed on nearly all primary human tumors of epithelial origin (Groh, et al., 1999), and RAET1 ligands are expressed on many human tumors as well (Cosman, et al., 2001). Rae1 and H60a have been shown to be upregulated in the skin after exposure to a carcinogen (Girardi, et al., 2001).

NKG2D ligands are also upregulated on cells infected with certain pathogens (Groh, et al., 2001, Tieng, et al., 2002). One study showed that stimulation with toll-like receptor ligands upregulates Rae1 transcripts in mouse macrophages, but whether this results in cell surface expression has been controversial (Hamerman, et al., 2004), unpublished observations).

The mechanisms responsible for induction of NKG2D ligands in cells exposed to different forms of stress are not fully understood, but studies to date have provided important insights.

DNA-damaging agents or DNA replication inhibitors have been shown to upregulate NKG2D ligands in an ATM and/or ATR dependent manner (Gasser, et al., 2005). ATM and ATR are the key initiators of the DNA damage response pathway. The pathway has not been fully worked out, but unpublished data show that Rae1 induction in cells exposed to DNA damage is due to stabilization of the *Raet1* mRNA as opposed to transcriptional activation or protein-level regulation (B. Hsiung and D. Raulet, unpublished data). An activated DNA damage response is characteristic of precancerous lesions and some advanced cancers (Bartkova, et al., 2005, Gorgoulis, et al., 2005), suggesting that the activation of the DNA damage response by oncogenic stress can culminate in the induction of NKG2D ligands and a host immune response against the cancer cells (Gasser, et al., 2005). Recent evidence indicates that the ligand for another activating receptor on NK cells, DNAM-1, is also induced by the DNA damage response, dependent on ATM- and ATR (Soriani, et al., 2009). These studies show that the DNA damage checkpoint response, which is regarded as an intrinsic barrier to tumorigenesis, can also activate an immune response.

Interestingly, cell senescence, which restricts tumorigenesis by inhibiting proliferation, has also been linked to immune cell- mediated tumor clearance. In a study of liver tumors arising in cells lacking p53, it was found that reactivation of p53 expression in these cells in vivo resulted in senescence and the eventual clearance of the senescent cells. The clearance of the cells was prevented by depleting NK cells, macrophages or neutrophils from the mice, demonstrating that it was immune mediated (Xue, et al., 2007). Another study of senescent cells showed that NKG2D ligands, MICA, ULBP2 are induced in vitro upon p53 re-activation (Krizhanovsky, et al., 2008). These studies suggested that the senescence program during tumorigenesis could be associated with the induction of NKG2D ligands.

Another well-known stress pathway, the heat shock response, was reported to upregulate MICA/MICB transcription (Venkataraman, et al., 2007) as a result of heat shock response elements in the promoters. Heat shock did not induce NKG2D ligand transcripts in studies of mouse cell lines (Gasser, et al., 2005). However, heat shock and UV stress post-translationally induced expression of one NKG2D ligand in mice, Mult1, by inhibiting ubiquition-dependent degradation of the protein (Nice, et al., 2009).

Recently, a distinct type of stress that regulates H60c on epidermal cells was reported. When the skin is wounded, or when keratinocytes from the skin are cultured in high oxygen, H60c transcript levels were induced (Whang, et al., 2009). This induction may play a role in stimulating skin-resident immune cells, such as dendritic epidermal T cells, which express NKG2D constitutively. DETC are activated as a result of skin wounding and their function has been reported to be essential for normal healing of skin wounds (Jameson, et al., 2002).

Another study reported that Rae1 expression is inhibited by the transcription factor JunB, an AP-1 subunit (Nausch, et al., 2006). The absence of *JunB* resulted in induction of Rae1 protein and mRNA expression. As will be discussed later, JunB is known to inhibit $p16^{INK4a}$, which is a negative regulator of cell cycle (Passegue and Wagner, 2000), meaning that inhibition of JunB could result in increased cell proliferation. These considerations may be very relevant to the findings reported in this dissertation, as will be discussed later.

Despite the findings reported here concerning the mechanisms of NKG2D ligand induction, much remains to be learned. For example, next to nothing is known concerning the regulation of transcription of genes encoding NKG2D ligands. Moreover, some findings indicate that the present understanding cannot fully account for the induction of NKG2D ligands in the context of cell cultures or in vivo tumorigenesis. For example, DNA damaging agents are often not sufficient to strongly induce NKG2D ligands in certain primary cultured cells, such as cultured lymphocytes. As another example, a study of tumorigenesis in the EuMyc transgenic model of B lymphomagenesis showed that the DNA damage checkpoint is activated in the precancerous Myc-expressing B cells of relatively young mice, but no NKG2D ligands are expressed until a later stage when lymphoma cells become detectable (Unni, et al., 2008). Therefore it is apparent that we currently lack a comprehensive understanding of the factors that regulate NKG2D ligand expression.

NKG2D and immunesurveillance

Evidence has accumulated showing that NKG2D has an important role in the immunesurveillance of tumors. Tumor cell lines are subject to cytolysis when the cells express NKG2D ligands *in vitro* (Bauer, et al., 1999, Jamieson, et al., 2002, Cerwenka, et al., 2000, Diefenbach, et al., 2000). In the case of rare cell lines that do not express NKG2D ligands, introduction of such ligands by transduction results in the cells becoming highly sensitive to elimination by NK cells and in some cases T cells in vivo after subcutaneous transfer (Diefenbach, et al., 2001, Cerwenka, et al., 2001). Recently reported NKG2D deficient mice provided the most direct support for NKG2D's role in immunesurveillance in vivo. In mice containing a transgene that causes prostate cancer (TRAMP- mice), the incidence of an aggressive form of adenocarcinoma was significantly higher in NKG2D-deficient mice compared to littermate controls. More strikingly, TRAMP tumors that arose in the absence of NKG2D retained expression of NKG2D ligands, whereas tumors from NKG2D+ mice had extinguished ligand expression (Guerra, et al., 2008), presumably due to selective destuction of cells expessing the ligands. These data strongly supported the role of NKG2D in immunesurveillance against cancer.

The impact of NKG2D on tumorigenesis was also investigated in the EuMyc model of lymphomagenesis. In this case, tumorigenesis was accelerated in NKG2D knockout mice, but NKG2D expression did not detectably influence whether the tumors that did arise expressed NKG2D ligands at the cell surface (Guerra, et al., 2008). These data suggest that some classes of tumors can evade NKG2D-dependent immunesurveillance without extinguishing expression of NKG2D ligands.

The means by which tumors may evade NKG2D recognition are of considerable interest. Several studies reported that sustained NKG2D engagement induces NKG2D receptor internalization, and ultimately leads to a broad impairment of NK cell functions, even those activated by receptors other than NKG2D (Oppenheim, et al., 2005, Wiemann, et al., 2005). Also, NKG2D ligands can be shed from the cell surface (Groh, et al., 2002). Shed ligands are found in the serum of cancer patients and correlate with a reduction of the amounts of NKG2D on the surface of NK and T cells. Thus, the combination of sustained NKG2D engagement by cell bound ligands and/or by shed ligands may desensitize NK cell functions globally, and this may represent one mechanism whereby tumors evade recognition by NKG2D. It is likely that other unidentified mechanisms also play a role in evasion of NKG2D recognition.

Regulation of proliferation

This dissertation concerns regulation of an NKG2D ligand in response to proliferative signals. Therefore, some introduction of the pathways regulating cell proliferation is necessary. Of particular relevance to my thesis is the role of the E2F family transcription factors, which have been implicated by my data in the regulation of NKG2D ligands in proliferating cells

Uncontrolled proliferation is a hallmark of cancer. In normal cells, multiple regulatory mechanisms exist to ensure that proliferation is tightly controlled.

Most fully differentiated cells *in vivo* remain healthy in the nonproliferating or quiescent state often called G0 (Pardee, 1989). Proliferative signals cause cells to enter the G1 phase of cell cycle. One of the most important regulatory factors at the G0-G1 transition (or perhaps in the entire cell cycle) is the retinoblastoma protein (pRB) - E2F transcription factor axis. For most of the G1 phase, pRB is hypophosphorylated, which enables binding to E2F. Binding of pRB to E2Fs inhibits transcription of E2F target genes involved in DNA replication and cell cycle progression (Chellappan, et al., 1991).

pRB is phosphorylated by CDK4 and CDK6, which are activated by cyclins of the D-class; D-cyclins are themselves induced by growth factor stimulation Phosphorylation of pRB blocks its inhibitory activity and therefore permits transcription of E2F target genes that are essential for cell cycle progression to S phase (Weinberg, 1995).

This pathway is cooperatively regulated by CDK inhibitors (CKIs). CKIs include INK4 proteins (Inhibitors of CDK4), $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c,}$ and $p19^{INK4d}$ that specifically inhibit the catalytic subunits of CDK4 and CDK6. In addition, the Cip/Kip family proteins inhibit the activity of cyclin D-,E- and A- dependent kinases. The Cip/Kip family includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (Sherr and Roberts, 1999). Deregulation of factors that control G1/S transition, which heavily relies on E2F activity, is a common theme in the process of tumorigenesis. Due to its critical role, pRB and the associated regulatory pathway is one of the most disregulated pathways in human cancer (Weinberg, 1995).

E2F family transcription factors

The E2F family consists of 8 transcription factors containing one or more conserved DNA binding domains that bind target promoters and regulate their expression (Chen, et al., 2009). The 8 E2F proteins are distinguished into two groups depending on whether they activate transcription (E2F1, E2F2, and E2F3a) or repress it (E2F4-8 and E2F3b). The 3 activating E2Fs exhibit a high degree of functional redundancy, as do the 5 repressors, making the study of these proteins challenging.

The activity of E2Fs is controlled by two DP and three RB related proteins (pRB, p107 and p130). The activating E2F proteins, E2F1, E2F3 and E2F3a interact exclusively with pRB. Overexpression of E2F activators can trigger quiescent cells to enter G1 phase independent of growth factor stimulation, possibly by overcoming inhibition mediated by pRB. The repressor function of E2F4-5, on the other had, involves the recruitment of p170 or p130 (pocket proteins) to E2F regulated promoters, which blocks transcription of target genes. Another group of repressors, E2F6-7, act independently of pocket proteins. The function of E2F8 is not fully understood (Dimova and Dyson, 2005).

This dissertation examines the activating function of E2Fs in proliferating cells. E2Fs control the expression of genes encoding various DNA replication proteins and cell cycle regulators. Well-known targets of E2F1 include cyclin D, cyclin E, CDKs, c-myc and E2F1-3. Gene expression microarray data demonstrated that 7% of human genes, and 2% of CpG island regions are induced directly or indirectly by the E2F family (Muller, et al., 2001, Weinmann, et al., 2002). Surprisingly, Chip-Chip analysis of high-density tiling arrays showed that 35% of human ENCODE genes are bound by E2F1 within the promoter region (Bieda, et al., 2006). ENCODE is the ENCyclopedia Of DNA Elements, consisting of regulatory sequences including DNase hypersensitive sites, CpG sites etc, and represent 1% of the human genome. Unexpectedly, not all targets of E2Fs are induced in a cell-cycle dependent manner, suggesting that there are multiple modes of E2F-regulation (Iwanaga, et al., 2006).

Several studies have suggested that E2F1 functions in the DNA damage response. It is known that the E2F1 protein is induced by DNA damage. Sensors of the DNA damage checkpoint pathway, ATM and ATR, were shown to phosphorylate E2F1, resulting in increased stability of the protein. E2F targets include a number of genes which function in DNA repair and recombination and DNA damage checkpoints (Dimova and Dyson, 2005). DNA damage-induced E2F1 activity as well as E2F1 overexpression can induce apoptosis (Stevens, et al., 2003). E2F1 increases the stability of p53 possibly by phosphorylation, and induces p53-dependent apoptosis. p53-independent E2F1 induced apoptosis is also proposed, mediated by p73, a member of p53 family (Urist, et al., 2004).

Because E2F1 increases transcripts of gene encoding DNA checkpoint, DNA repair and recombination proteins, it appears that E2f1 plays a substantive role as a mediator of some of the known outcomes of the DNA damage response.

Although the role of activator E2Fs in cell cycle entry is established, the role of E2Fs in cancer is not as clear. Ablation of E2F1 in $Rb1^{+/-}$ mice significantly reduced cancer incidence, resulting in a prolongation of the tumor-free lifespan of $Rb1^{+/-}$ mice (Yamasaki, et al., 1998). Another study showed that in a model of SV40 T antigen-induced neoplasia, E2F2-deficiency resulted in reduced epithelial cell proliferation (Saenz-Robles, et al., 2007). Although these findings support the role of E2Fs in tumorigenesis, other studies are more consistent with its function as a tumor suppressor. One study showed that $E2F1^{-/-}$ mice develop lymphomas or (less frequently) tumors of

mesenchymal origin (Yamasaki, et al., 1996). Another study showed that E2f2-deficiency in mice increased the incidence of Myc-induced T cell lymphomas, and that reintroduction of E2F2 into these tumours stimulated apoptosis of the tumor cells. These findings provide examples of an E2F activator, functioning as an oncogene or as a tumour suppressor in a tissue and context-specific manner.

In human cancer, the levels of E2Fs are increased in a large number of tumors. However, deletions of the E2f gene have been detected in some cancers, including thyroid cancer, pancreatic cancer and neuroblastoma. Overall, E2Fs are generally increased in cancers and have a role in supporting uncontrolled proliferation. But whether deregulated expression of E2Fs itself can induce tumorigenesis has not been shown.

Chapter2. Materials and Methods

Mice

C57BL/6J (B6) mice were bred in the UC Berkeley Animal Facility from breeders obtained from the Jackson Laboratory (Bar Harbor Me) and renewed with new breeders every other generation. *Nkg2d*$^{-/-}$ mice (officially called *Klrk1*$^{-/-}$ mice) were generated in our laboratory in the B6 genetic background (Guerra, et al., 2008). *p53*-/-, *p19*^{ARF}-/- and *p16*^{INK}/*p19*^{ARF}-/- mice were obtained from the National Cancer Institute Animal Repository (Frederick, MD).

Primary adult fibroblast preparations

Fibroblasts were prepared from tail segments from B6 wild-type mice or some cases *p73*-/- mice (gift from Dr. E Flores, University of Texas, MD Anderson cancer center) or ATR floxed mice (gift from Dr. E. Brown, University of Pennsylvania). For preparation, at least 3~4cm segment of tail is cut, incubated in 70% ethanol for 10 minutes to sterilize the tissue. Air-dried tail segments were washed with medium supplemented with 10% fetal calf serum (FCS). Skin and hairs were stripped off by pulling scissors along the tail tissue and cut into small pieces at the natural juctions. Tail pieces were incubated in 2.2ml enzymatic solution per tail (2 ml of a stock of 160 U/ml collagenase 1 (Sigma) in McCoy's 5A +0.2 ml of a stock of 2.5 mg/ml pronase (Roche) :Stock solution prepared by first dissolving pronase to 20 mg/ml in 0.01M tris pH 7.8 + 0.001M EDTA, and self-digesting at 37°C for 30~60minutes. Then dilute to 2.5 mg/ml) at 37°C for ~75minutes (60~90minutes) on a rocker.After the incubation period, the tube was filled with ice-cold medium and centrifuged 1200~1500rpm for 10minutes at 4°C to wash. The pellet was resuspended and suspension was passed through sterile nylon mesh to eliminate bone fragments. The flow-through was pellet down and resuspend in 10ml of complete DMEM containing amphotericin B (fungizone) at a final concentration of 2.5µg/ml. The cells were passaged after ~72 hours.

Cell lines and cell preparations

Fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated FCS, 2mM glutamine, 25mM HEPES, 1mM sodium pyruvate, 100uM non-essential amino acids, 100U/ml penicillin and streptomycin and 50uM 2-mercapto-ethanol (DMEM/FCS). The RMA mouse T lymphoma cell line and its derivatives, RMA-Rae1, RMA-Rae1-OVA were cultured in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated FCS, 2mM glutamine, 25mM HEPES, 50uM 2-mercapto-ethanol, 100U/ml penicillin and streptomycin (RPMI/FCS). Lymphokine activated killer cells (LAKs) were prepared by incubating B6 splenocytes for 4 days in RPMI/FCS supplemented with

1000U/ml recombinant IL-2 (rIL-2) in T125 flask at 2 X 10^6cells/ml. For splenocyte preparation, single cell suspension of splenocytes were treated with ACK lysis buffer for 1 minute, followed by 2X wash. Cells were passaged under white light.

Plasmids

The pGL3-*Raet1e* promoter constructs were generated by Dr. Ben Hsiung, Joseph Chavarria or myself. The promoter fragments were inserted between HindIII and XhoI site of the pGL3 vector (lacking an enhancer or promoter). The E2F1 binding site (-56, -10) mutants were made by site-directed mutagenesis (Quickchange, Stratagene). The E2F binding sites core sequence SSCGC was substituted with ATATC. Primer sequence used to generate E2F binding site mutant is following:

mut1 5' CCTCCTCCCTGGCTTCATATCGGGCGGCGCCTTCCG
mut1 3' CGGAAGGCGCCGCCCGATATGAAGCCAGGGAGGAGG
mut2 5' CATTGTGTCCATCCCCTGATATCAGAGCGCCGCTTGCCCG
mut2 3' CGGGCAAGCGGCGCTCTGATATCAGGGGATGGACACAATG

pcDNA-CMV-E2F1 (a gift from Dr. Farnham) was decribed previously (Li, et al., 1994). pCMV6-E2F2 was purchased from Origene. pCX-c-Myc plasmid was purchased from Addgene. pBABE-E2F1, E2F3, pMSCV-E2F2 were gifts from Dr. Stephan Gasser .

Plasmid transfection and retroviral transduction

Plasmids were transfected into fibroblasts using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) using 0.8µg DNA total and 2µl of lipofectamine per wells in 24well plates and the media was changed to either DMEM+FCS10% or 0% after 4-6 hours.

For retroviral transduction, retroviral supernatants were generated by cotransfecting 293T cells with plasmids encoding VSV gag/pol, env and pMSCV or pBABE retroviral constructs using Lipofectamine 2000 (Invitrogen). Culture supernatants collected 48 h after transfection were added directly to actively proliferating fibroblasts. RNA was harvested 48h after transduction using TRIZOL.

Dual luciferase reporter assay

Cells were plated at a density of $5X10^4$ cells per well in 24 well plates and were transfected 24 h after plating with 50 ng reporter construct, 0.1 ng pRL (Renilla reporter plasmid) and increasing amounts of pcDNA-E2F1 (100 – 750ng). The total mass of transfected DNA in each well was kept constant by adding empty vector plasmid DNA, when necessary. All experiments were performed in

triplicate, and data are presented as means ± SD. The Dual-Luciferase® Reporter Assay System (Promega) was used to measure luciferase luminescence. Luminescence was measured using LMAXII[384].

Statistical Analysis

All statistics were analyzed on Prism, using Student's T-test. *=$p<0.05$, **=$p<0.05$, ***=$p<0.005$

Nuclear run-on transcription assay
modified from (Core, et al., 2008)

For nuclear run on, at least $5X10^6$ cells were used per condition. To isolate the nuclei, the cell pellets were incubated with 5ml swelling buffer (10mM Tris-HCl pH7.5, 2mM $MgCl_2$, 3mM $CaCl_2$) and incubated in 4°C for 5 minutes, centrifuged, followed by resuspending with lysis buffer (swelling buffer + 0.5% NP-40 + 2U/ml Superase In (Ambion) + 10%glycerol) and pipetted 20~30 times to open up the membrane. Washed with lysis buffer and the pellet was resuspended with 100µl of freezing media (50mM Tris-HCl (pH8.3), 40% (v/v) Glycerol, 5mM MgCl2, 0.1mM EDTA+ 10X protease inhibitor (complete mini, EDTA-free, Roche, 11836153001 (Added right before the experiments)). 100µl nuclei solution was mixed with 0.1ml reaction buffer (10mM Tris-HCl pH8.0, 5mM MgCl2, 300mM KCl, 0.5mM ATP/CTP/GTP and Br-UTP (Invitrogen)) and incubated at 30°C for 15minutes. Then, 28µl DNase1 buffer and 60µl of DNaseI (Promega RQ1 DNase 1-Cat# M6101) was added to the mixture and the reaction was incubated for another 30minutes at 37°C. The reaction was quenched by adding Trizol and the RNA was extracted according to the manufacturer's instructions.

Immunoprecipitation of Run-on transcribed RNA

BrdU antibody / agarose complex (IIB5-AC, Santa Cruz) ,which cross-react with Br-UTP, was blocked with with 0.1% Polyvinylpyrrolidone (PVP) and 1µg/ml Bovine Serum Albumin (BSA) in 5X volume binding buffer. Run-on transcribed RNA was heated to 70°C for 5 minutes and placed on iced for 2 minutes, followed by incubation with antibody/bead complex in binding buffer (0.5XSSPE, 1mM EDTA, 0.05% Tween) for 30~60minutes. Following extensive washing (5X with different buffers; binding buffer, low salt buffer (0.2X SSPE, 1mM EDTA, 0.05% Tween), high salt buffer (low salt buffer + 137.5mM NaCl) and 2X TET buffer (TE +0.05% Tween), the RNA-antibody complex was eluted 4X with 125µl of elution buffer (20mM DTT, 300mM NaCl, 5mM Tris-HCl pH 7.5, 1mM EDTA, and 0.1% SDS) heated to 37°C, each 5 minutes, vortexed every minutes and extracted by TRIZOL (Invitrogen).

Quantitative RT-PCR

Total cellular RNA was prepared from the indicated tissues and cells using TRIZOL reagent (Invitrogen), followed by digestion of contaminating DNA using DNA-free (Ambion) according to the manufacturer's instructions. RNA was reverse transcribed using Superscript III reverse transcription (Invitrogen), and the resulting cDNA was used for qPCR. Triplicate amplification mixtures were prepared with 0.1–1 μg cDNA, SYBR GreenER SuperMix (Invitrogen), and 200 nM forward and reverse primers, and cycled using the ABI 7300 Real-Time PCR system. Cycling parameters used were 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 s and of 60°C for 60 s. The following primers were used:

Raet1e forward 5' CAGGTGACCCAGGGAAGATG 3'
reverse 5' CTCAACTCCTGGCACAAATCG 3'
Unspliced Raet1e forward 5' ATTTGTGCCAGGAGTTGAGG 3'
reverse 5' CCTGCATGTACTCTGCCCTT 3'
E2F1 forward 5'GCCCTTGACTATCACTTTGGTCTC 3'
reverse 5'CCTTCCCATTTTGGTCTGCTC 3'
E2F2 forward 5'GCCACCACCTACTACACTTCG 3'
reverse 5' CGGAATTCAGGGACCGTAG 3'
Myc forward 5'CAGAGGAGGAACGAGCTGAAGCGC 3'
reverse 5' TTATGCACCAGAGTTTCGAAGCTGTTCG 3'
18s forward 5' GTAACCCGTTGAACCCCATT 3'
reverse 5' CCATCCAATCGGTAGTAGCG 3'
cyclin E forward 5' TTGTGTCCTGGCTGAATGTCTATGTCC 3'
reverse 5' CTGCTCGCTGCTCTGCCTTCTTACT 3'

Chromatin Immunoprecipitation

Proliferating or 3 day serum starved fibroblasts that had been serum-starved for 3 days were harvested with trypsin and resuspended in DMEM 10% FCS. Formaldehyde fixing solution (0.1M NaCl 1mM EDTA (pH 8.0) 0.5mM EGTA (pH 8.0) 50mM Hepes (pH 8.0) 11% formaldehyde) was added to the medium to achieve a final concentration of 1% formaldehyde. Cross-linking was allowed to proceed for 10 minutes at room temperature and then stopped by the addition of glycine to a final concentration of 0.125 M, followed by washing with cold PBS twice. Lysates were sonicated to shear the genomic DNA into fragments of between 200 and 600 bp (30% amplification, 20s, 8~10X). The day before sonication, polyclonal antibodies specific for either E2F1 (KH95, Santa Cruz Biotechnology), E2F2 (C-20, Santa Cruz Biotechnology), E2F3 (C-20, Santa Cruz Biotechnology) or c-Myc (N-262, Santa Cruz Biotechnology) were blocked with and incubated with Dynal beads (Invitrogen) overnight at 4°C. Sonicated samples were immunoprecipitated overnight at 4°C with antibody-

beads complexes. Following extensive washing with 4 different buffers (3X with Low Salt Wash Buffer (1st wash in 5 ml and the rest in 1mL) 1X with High Salt Wash Buffer 1X with LiCl Wash Buffer 2X with TE Buffer), the complexes were eluted by incubating elution buffer (10mM Tris (pH 8.0)1mM EDTA 1% SDS) at 65C for 30minutes and the cross-links were reversed by adding NaCl final to a final concentration of 0.2M and heating the samples to 65°C overnight. The eluted material was phenol-chloroform extracted, ethanol precipitated, and resuspended in 50µl of water. I observed a minimal amount of PCR product for the mock and no-antibody control reactions. The following primers were used:
Raet1e promoter
forward 5'- GGCTGTAATTTGCACACTCG - 3'
reverse 5'- GGAGCAGGTAACTGACTACAGC - 3'
p107 promoter
forward 5'- TTAGAGTCCGAGGTCCATCTTCT - 3'
reverse 5' - GGGCTCGTCCTCGAACATATCC - 3'

Antibodies and Flow Cytometry

Pan-Rae1 specific MAb (186107), RAE-1 ε-specific MAb (205001), and Mult1-specific MAb (237104) were purchased from R&D Systems for staining NKG2D ligands. FITC-conjugated CD45 antibody (30-F11) was purchased from eBioscience and Biotin-conjugaed BrdU antibody (MoBU-1) was purchased from BioLegend. BrdU staining was performed using the BrdU flow kit (except antibody) following the manufacturer's instructions (BD Pharmingen, San Jose, CA).

Cytotoxic Assay

Target cells were labeled with 50 µCi of $Na_2(^{51}Cr)O_4$ for 60 minutes at 37°C in RPMI-1640 medium containing 10% FCS, and washed three times with medium. ^{51}Cr-labeled target cells (10^3) and effector cells were mixed in U-bottomed wells of a 96-well microtiter plate at the indicated E/T ratios in triplicate. After 4 hr incubation, the cell-free supernatants were collected, and the radioactivity was measured with a Micro-β counter (Wallac, Turku, Finland). The spontaneous release was in all cases less than 20% of the maximum release. The percentage of specific ^{51}Cr release was calculated according to the following formula: % specific lysis = (experimental − spontaneous) release × 100/(maximal − spontaneous) release.

Immunofluorescence

Tissues were frozen in O.C.T. freezing medium (Tissue-Tek) and cut into 7~10 µM sections. Sections were fixed with cold acetone for 15 minutes and blocked

with 2.4G2 for 15minutes followed by 10% donkey serum for 1 hour at room temperature. The slides were stained with RAEε antibody (1:100) overnight. For BrdU staining, sections were fixed with acetone, and incubated in HCl (1 M) for 10 minutes on ice, followed by incubation with HCl (2 M) at room temperature for 10 minutes, then 20 minutes at 37°C to open up DNA structure. Immediately after acid incubation, the sections were neutralized by incubation with borate buffer (0.1 M) for 10 minutes at room temperature, followed by washing three times with 0.1% triton X-100.
Coverslips were mounted on slides with vectashield with DAPI (Vector Labs) and the fluorescent signal was visualized using a Nikon eclipse E800 microscope.

Wound healing

Mice were anesthetized with isoflurane. The mouse backs were shaved, back skin was pulled up and a 2-mm punch tool was used to create 4 sets of wounds to generate 8 holes, as previously described (Jameson, et al., 2002). Wounds were left uncovered. For wound size measurement, wounded mice were sacrificed 0-3 days after wounding and the whole back skin was peeled off. Skin was laid flat on a culture dish filled with DMEM, and all skins were photographed at an equal distance from the wound (20 cm). Wound sizes of the wounds were measured using the ImageJ program by 'freehand shaping' the wound area. As skin fragments from wounding were sometimes not completely detached and overlapped, 4 out of total 8 wounds, which were not fragmented and overlapped, were measured and averaged. At least two mice were used per time point. In some of the experiments, one day before sacrifice, mice were injected intraperitoneally with 50 mg/kg BrdU.

RNA preparation from wounded skin

Wounded mice were sacrificed 1-4 days after wounding and the skin was excised including the border around the wound. The excised skin was flash frozen with liquid nitrogen, ground with a mortar and pestle and total cellular RNA was extracted using TRIZOL (Invitrogen) according to the manufacturer's instructions.

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Tumor injection and measurement

RMA-Thy1.1, RMA-RAE1b-Thy1.1, RMA-OVA-Thy1.1 and RMA-Rae1b-OVA-Thy1.1 were generated by Dr. Sophie Lehar in the Raulet lab. RMA T lymphoma cells line was obtained from James Allison's lab. Indicated number of RMA cells and its derivatives were injected subcutaneously into B6 mice and the tumor volumes were determined on the dates indicated by measuring width X height X depth using a caliper. In some experiments, splenocytes from OT-1

transgenic NKG2D WT or KO mice were intravenously injected into B6 mice 2 days prior to tumor injection.

Chapter 3. Expression of RAE-1 ε is linked to cellular proliferation

RAE-1 ε expression is induced in freshly prepared fibroblasts in culture

To date, NKG2D ligand regulation had been studied primarily with cell lines that had been maintained for long periods in culture, which is known to induce multiple mutations in key stress regulators including p53, $p19^{ARF}$ or $p16^{INK4a}$ (Kamijo, et al., 1997). To investigate the regulation of NKG2D ligands in cells that retains normal checkpoint pathways, fibroblasts were freshly prepared from the adult B6 mouse tails and cultured *in vitro*. Unexpectedly, RAE-1 ε was detected on the surface of fibroblasts within 2 days of culture without any additional treatment and the level of expression continued to increase until day 6 when it reached a plateau (Fig. 3-1). Notably, RAE-1 ε and a small and variable amount of Mult1, but not other RAE1 isoforms was detected in B6 fibroblast primary cultures (data not shown). RAE-1 ε was induced similarly on fibroblasts cultured from other sources, as well, including the peritoneal wall and ear tissue (data not shown). Induction of RAE-1 ε at the cell surface was accompanied by induction of *Raet1e* mRNA in the cells (see Chapter 4).

The DNA damage response is not required for induction of RAE-1 ε

Previous studies have shown that the DNA damage response initiated by the activation of the ATR and ATM protein kinases induces cell surface expression of ligands for NKG2D on long-term cultured fibroblasts (Gasser, et al., 2005), Unpublished data) suggest that induction of RAE1 in these cells mediated by the DNA damage response occurs as a result of stabilization of the *Raet1* mRNA (Hsiung unpublished). To test whether the DNA damage response pathway is activated in primary cultured fibroblasts, freshly prepared fibroblasts were grown on tissue culture treated glass slides and stained with anti- γH2AX antibody, which is one of the key markers for activation of the DNA damage checkpoint response (Fig. 3-2. *A*). In day 5 primary cultured fibroblasts, clear γH2AX foci were observed in the nuclei, which showed that DNA damage response pathway was activated. To investigate whether the activation of the DNA damage response pathway is required for induction of RAE-1 ε in primary cultured fibroblasts, cells were exposed to a combination of DNA damage response pathway inhibitors, including SB218078, a Chk1 inhibitor and KU55933, an ATM inhibitor (Fig. 3-2. *B*). However, the inhibitors failed to block RAE-1 ε expression. Furthermore, tail fibroblasts carrying a conditional ("floxed") ATR allele, which were transduced with a Cre-expressing retrovirus to delete the ATR gene, induced RAE1 normally even when they were also cultured in the presence of the ATM inhibitor, KU55933 (Fig. 3-2. *C*). Taken together, these data established that although the DNA damage checkpoint response is activated in the cultures, the ATR and/or ATM-dependent DNA damage response pathway is not required for RAE-1 ε induction in primary cultured fibroblasts.

Role of p53 family, $p19^{ARF}$, $p16^{INK4a}$

When cells are stimulated to proliferate with high levels of growth factors or as a result of oncogene activation, tumor suppressor proteins are often induced to constrain the uncontrolled proliferation and eventually induce senescence or apoptosis. It was previously shown that the tumor suppressor genes, p53, $p19^{ARF}$ and $p16^{INK4a}$ are induced during early passages of primary cultured fibroblasts, and remain induced until one or more of these tumor suppressors are mutated or lost by another mechanism (Zindy, et al., 1998, Kamijo, et al., 1997). Therefore, we asked whether those tumor suppressor gene were involved in RAE-1 ε induction. To test this, primary cultured fibroblasts were prepared with tail samples from mice with deletions of genes for p53, the p53 homolog p73, $p19^{ARF}$ or both $p19^{ARF}$ and $p16^{INK4a}$. Five-10 days later, the cultured cells were stained for RAE-1 ε expression. Primary cultured fibroblasts that lacked p53, p73, $p19^{ARF}$ or $p16^{INK4a}$ expressed amounts of RAE-1 ε that were indistinguishable from the amounts on fibroblasts from wild-type littermates (Fig. 3-3). These data suggested that the tumor suppressor genes tested are not required individually for RAE-1 ε induction.

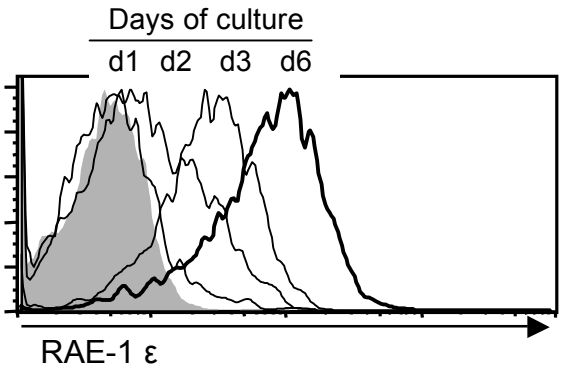

Fig. 3-1. RAE-1 ε is induced on the surface of proliferating fibroblasts.
Freshly prepared B6 adult mouse tail fibroblasts are harvested after 1~6 days of *in vitro* culture and stained with anti-RAE-1 ε antibody for flow cytometry analysis. Note that the expression of RAE-1 ε is significantly induced by culture over the isotype control (shaded histogram). Data are representative of at least five independent experiments.

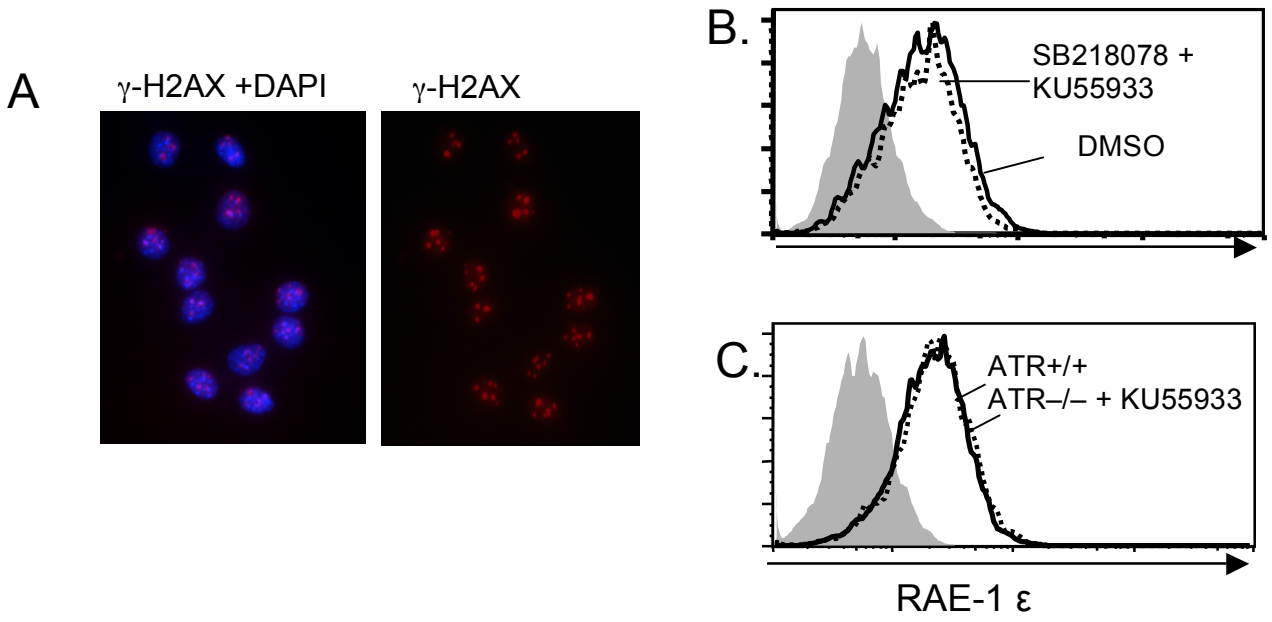

Fig. 3-2. The DNA damage response is not required for induction of RAE-1 ε induction. A. Immunofluorescence of fibroblasts after 5 days in primary culture. γ-H2AX staining is shown in red, indicating activation of DNA response pathway, DAPI in blue for nuclei. **B**. Fibroblasts were treated with Roscovitine or SB218078 (Chk1 inhibitor)+KU55933 (ATM inhibitor) for 3 days. **C.** Fibroblasts freshly prepared from ATR+/+ or ATR-/- mouse tail is cultured with DMSO or KU55933. Note that both histogram overlapped, indicating DNA damage response pathway inhibition did not affect the RAE-1 ε induction. Shaded histogram represents isotype control staining.

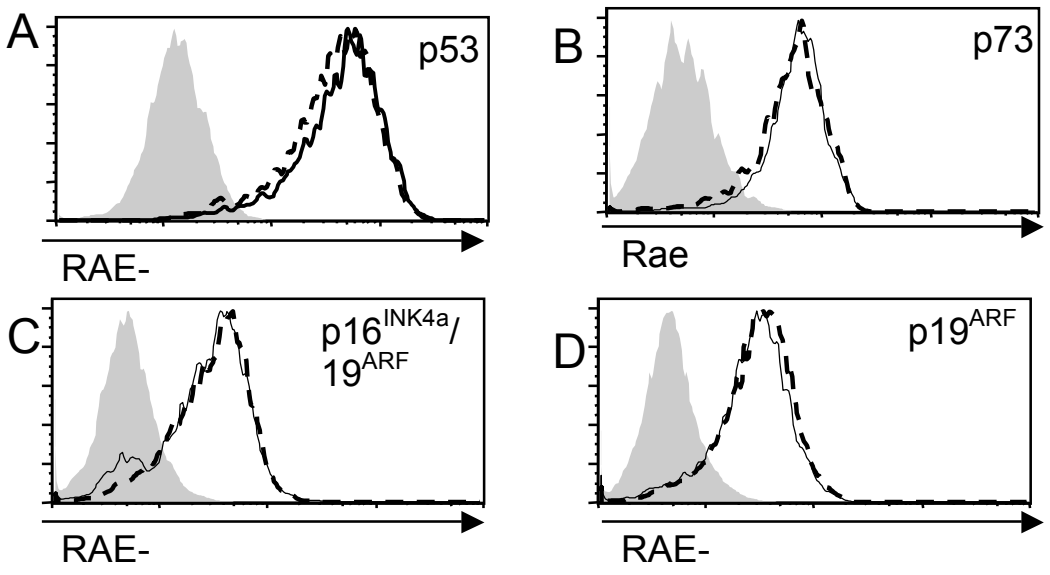

Fig. 3-3. Tumor suppressor genes, p53, p73, $p16^{INK4a}$ and $p19^{ARF}$ are not responsible for RAE-1 ε induction. Fibroblasts were freshly prepared from WT mice or mice that lack the indicated genes and cultured for 10 days (**A**) or 5 days (**B, C, D**). Note that the solid line which represents wild-type and the dashed line representing the indicated knockout are significantly different. Shaded histogram represents isotype control staining.

Oxidative stress is not responsible for RAE-1 ε *induction*

Another well-known stress pathway that is activated during cell culture is oxidative stress. Murine fibroblasts cultured in atmospheric oxygen (20%) were shown to accumulate oxidative DNA damage and undergo replicative senescence after one month in culture (Parrinello, et al., 2003). To examine whether oxidative stress caused by high levels of reactive oxygen species (ROS) induces RAE-1 ε, freshly prepared fibroblasts were exposed for 3 days to different doses of the ROS scavenger NAC (N-Acetyl Cysteine). As shown by staining with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCFDA), which detect the level of ROS, NAC effectively blocked ROS accumulation, RAE-1 ε expression was also significantly downregulated in NAC dose-dependent manner (Fig. 3-4. *A*). Although this finding initially suggested a role for ROS in RAE-1 ε induction, it was subsequently determined that cell proliferation, measured by population doubling was also severely inhibited by NAC treatment (Fig. 3-4. *B*), consistent with reports in the literature (Irani, et al., 1997). This observation made it hard to separate the effects of ROS versus proliferation in the induction of RAE-1 ε.

As an alternative approach to the question, conditions were adjusted to achieve low oxidative stress without the non-specific effects resulting from NAC treatment. A previous report demonstrated that fibroblasts primary cultured under a low concentration of oxygen (3%) failed to accumulate oxidative DNA damage and contained less ROS, but proliferated better than cells cultured in atmospheric concentrations of oxygen (~20%), at least in part because the cells failed to activate the p53 checkpoint (Parrinello, et al., 2003, Busuttil, et al., 2003). Therefore, primary cultured fibroblasts were established in the presence of either 3% or 20% O_2, and ROS levels and Rae1 induction were tested 3 days later. Although cells cultured in 3% O_2 contained lower amount of ROS as determined by DCFDA, RAE-1 ε expression was identical to that of cells cultured in 20% O_2 (Fig. 3-4. *C*). These data strongly suggest that oxidative stress is not responsible for RAE-1 ε induction.

Serum components other than growth factors are not required for Rae1 induction

In vivo, fibroblasts are normally sequestered from serum, but are exposed to serum when tissue is injured or is undergoing remodeling (Chang, et al., 2004). Exposure of fibroblasts to serum in culture is reported to induce stress responses that cause the cells to senesce, and in some studies the senescence-inducing components in serum were separable from growth factors. In contrast, cells cultured in defined serum-free media containing purified growth factors proliferate without senescing for a longer period and accumulate fewer chromosomal abnormalities (Rawson, et al., 1991, Woo and Poon, 2004). To test whether RAE-1 ε induction required the action of serum factors, freshly prepared fibroblasts were cultured on fibronectin coated plates in a serum-free medium containing insulin, transferrin, selenium and High-density lipoprotein (HDL) supplemented with epidermal growth factor (EGF), as described (Rawson, et al., 1991, Woo and Poon, 2004) (Fig. 3-5 *B*). Cells cultured in the serum-

free medium induced RAE-1 ε to a comparable level as that induced in serum-supplemented medium. In contrast, cells that were not supplemented with EGF or were cultured on plates that were not coated with fibronectin did not proliferate and failed to induce RAE-1 ε on the surface, consistent with a requirement for proliferation for induction of RAE-1 ε (Fig. 3-5. *C, D*). These data indicated that serum-free medium is sufficient to induce RAE-1 ε, and therefore that Rae1induction is not dependent on other specific factors in serum.

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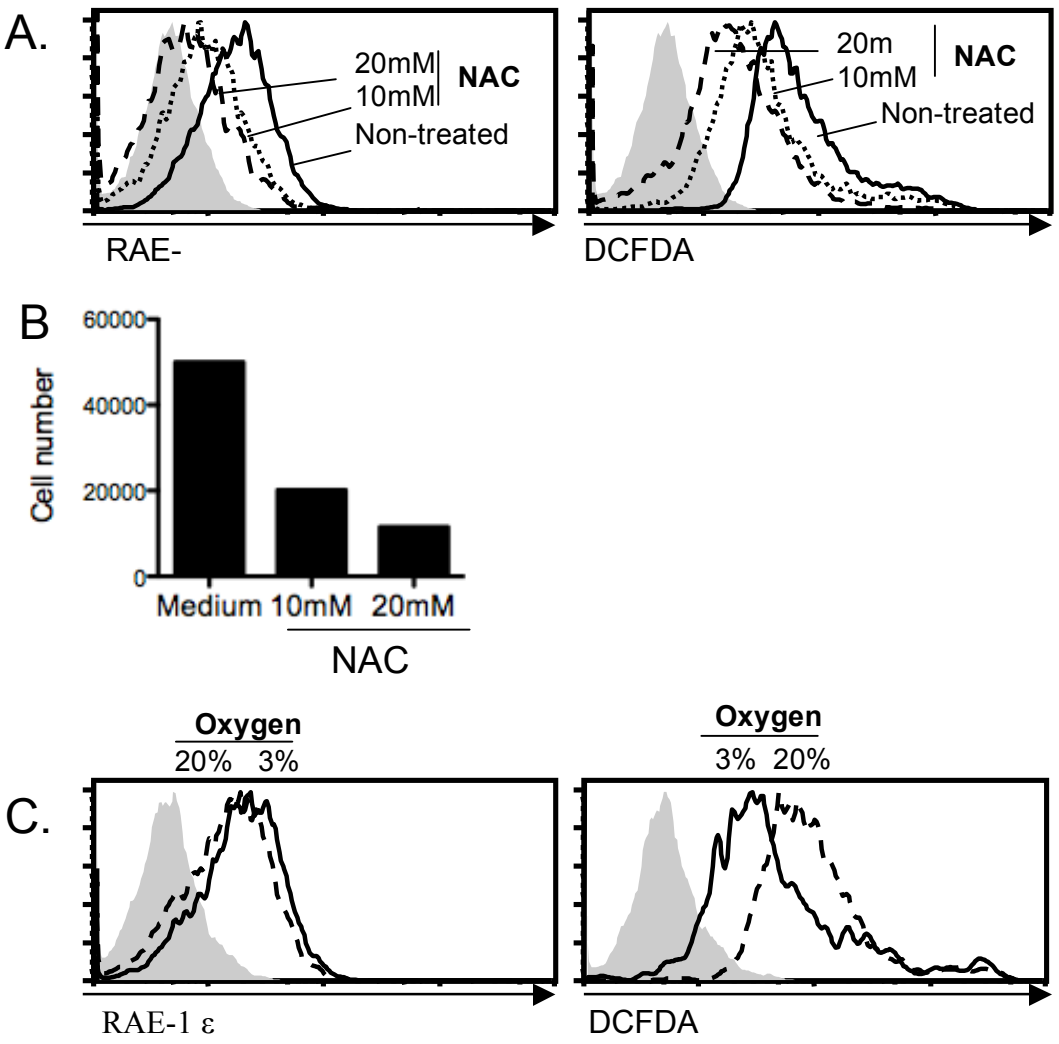

Fig. 3-4. Oxidative stress by ROS is not responsible for RAE-1 ε induction. Freshly prepared fibroblasts were plated at 10^4 cells/well in 24 well plate and treated with different doses of NAC (N-Acetyl Cysteine) for 3 days and (**A**) stained with RAE-1 ε antibody or (after permeabilization) with DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate) to measure ROS **B.** Cell numbers/culture were counted on day 3. **C.** Freshly prepared fibroblasts were cultured in 3% or 20% oxygen incubator for 3 days before staining as shown. Shaded histogram represents isotype control staining.

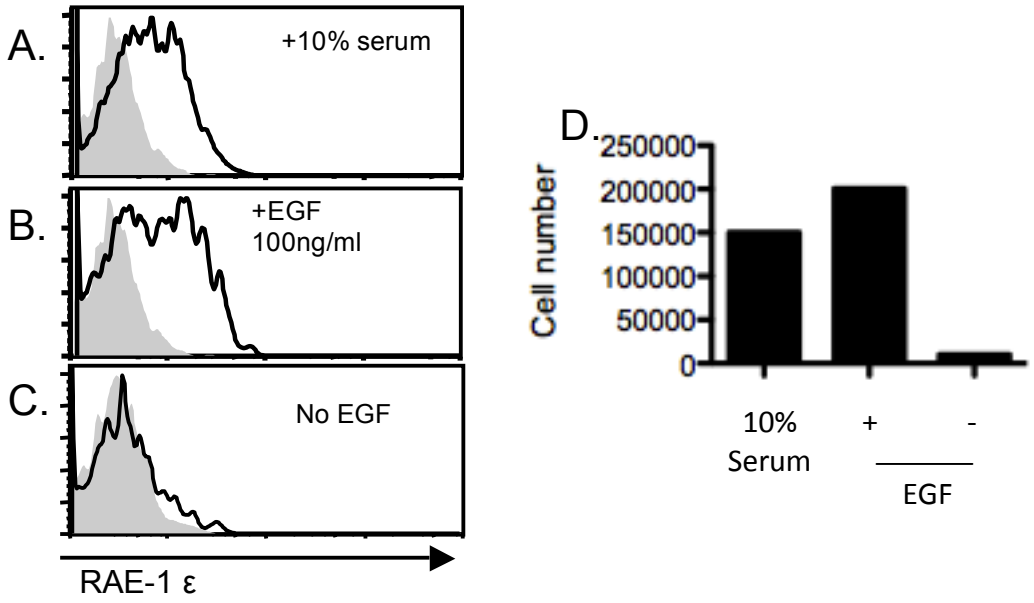

Fig. 3-5. RAE-1 ε is induced in serum-free cultures supplemented with growth factor. Freshly prepared fibroblasts were cultured in DMEM supplemented with 10% serum (**A**) or EGF (100ng/ml) on fibronectin coated plates (**B**) or without EGF (**C**) for 4 days. On day 4 cell numbers were counted before staining (**D**). All the cells examined in this figure were gated on 7-AAD negative cells to remove dead cells. Each panel in this figure depicts representative data from two or more independent experiments. EGF= Epidermal growth factor. Shaded histogram represents isotype control staining.

Proliferation inhibitors block RAE-1 ε *induction*

In order to investigate the pathways that spontaneously induce RAE-1 ε in primary cultures, freshly prepared fibroblasts were treated with an array of reagents. RAE-1 ε induction was significantly inhibited in the presence of inhibitors of the PI3K-mTOR pathway (LY294002 , Rapamycin), the MAPK pathway (SB202190, data not shown) or cyclin-dependent kinase (Roscovitine). All of these inhibitors severely decrease proliferation (Fig. 3-6. *A* and *B*) (Vlahos, et al., 1994, Marx, et al., 1995, Meijer, et al., 1997). These data, in combination with the results presented in Fig 3-3 and Fig 3-4, indicate that any treatments that inhibit pathways that induce or support proliferation are sufficient to block RAE-1 ε induction. These findings suggest that RAE-1 ε expression is closely linked to proliferation itself, as opposed to any of these specific pathways.

In contrast, drugs that either induce the DNA damage response (Aphidicolin, Cisplatin) or inhibit it (Chk1 inhibitor SB208078) did not alter RAE-1 ε expression, supporting the conclusion that the DNA damage is not involved in the induction process studied here.

Rae1 induction depends on growth factors in serum

To further confirm that proliferation is required for RAE-1 ε upregulation, freshly prepared fibroblasts were cultured in medium supplemented with 2% serum, which supports a slower rate of cell proliferation than 10% serum due to the lower concentration of growth factors in the medium. Compared to the rate of RAE-1 ε induction on cells cultured in 10% serum, there was a significant delay in RAE-1 ε induction on fibroblasts cultured in 2% serum (Fig. 3-7). As a more defined approach, fibroblasts were cultured in serum-free media supplemented with different doses of EGF. RAE-1 ε induced in a manner that depended on the dose of EGF, and which correlated with the level of induced proliferation (data not shown). These data demonstrated that RAE-1 ε induction is dependent on proliferation induced by growth factors.

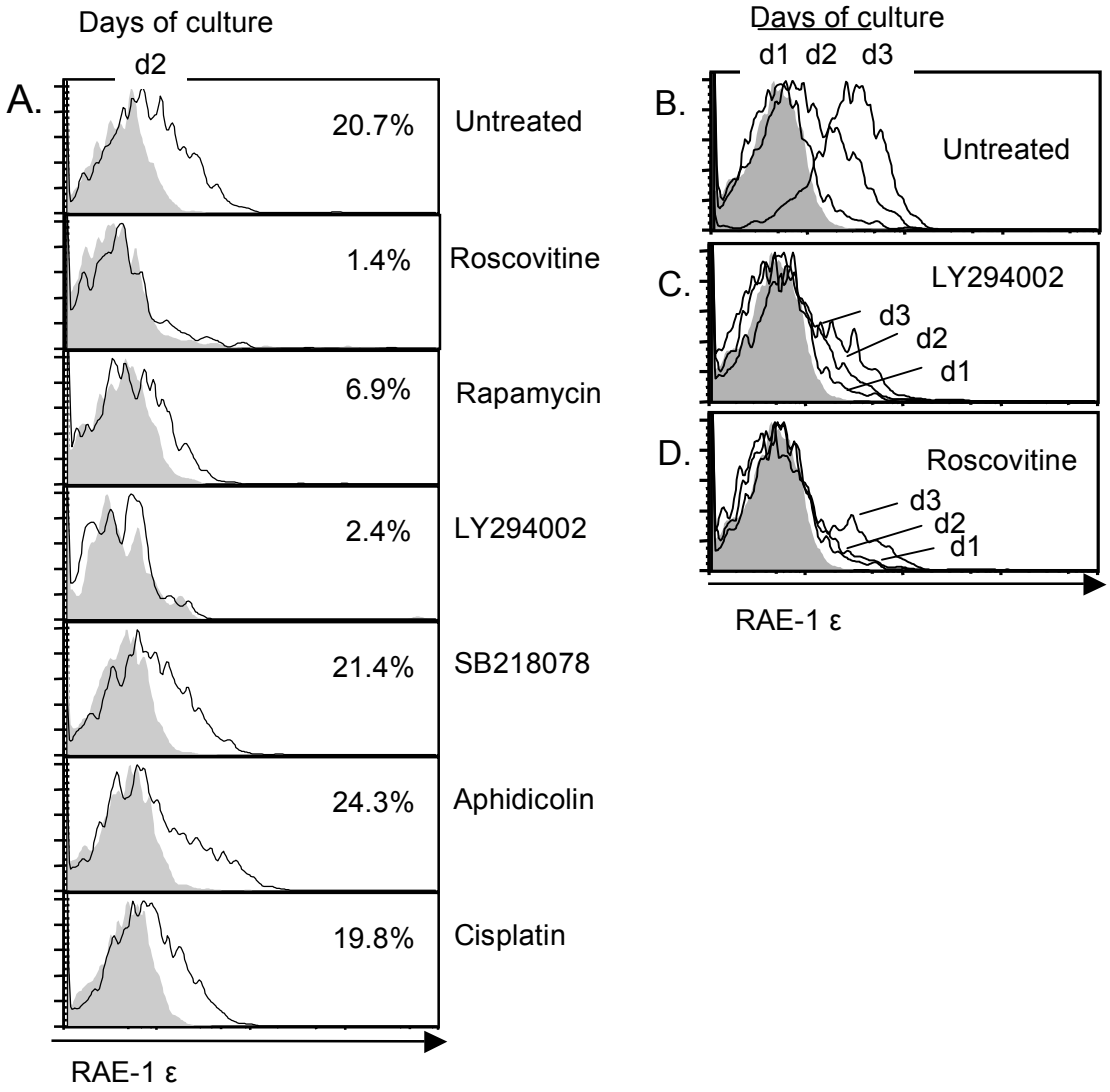

Fig. 3-6. RAE-1 ε induction is inhibited by anti-proliferative reagents . A. Freshly prepared B6 adult mouse tail fibroblasts were cultured for 2 days with DMSO (Untreated), Roscovitine (25 μM), Rapamycin (1 μM), LY294002 (10 μM), SB218078 (150nM), Aphidicolin (4 μM) or Cisplatin (10ug/ml). Numbers represents the percent RAE-1 ε positive cells. **B., C., and D** some of the cultures were maintained until day 3. All the cells examined in this figure were gated on 7-AAD negative cells to exclude dead cells from the analysis. Data are representative of at least four independent experiments. Shaded histogram represents isotype control staining.

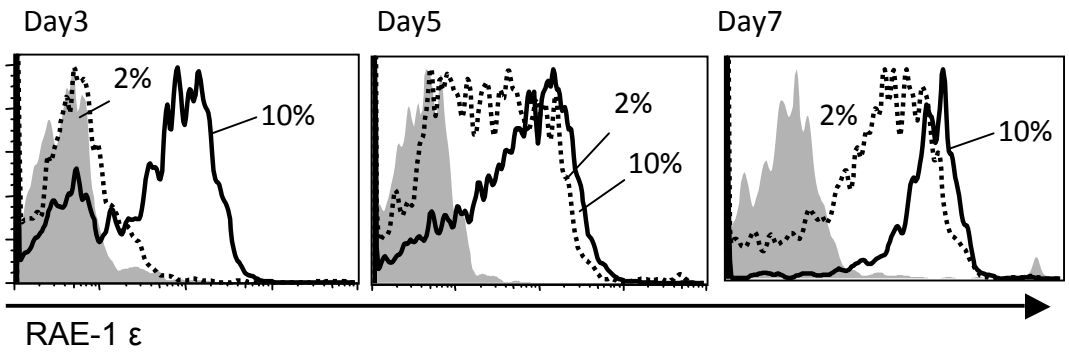

Fig. 3-7. RAE-1 ε expression is induced by growth factors in a dose-dependent manner. Freshly prepared B6 adult mouse tail fibroblasts are cultured in media supplemented with 2% or 10% serum for 3~7 days and harvested for flow cytometry analysis. All the cells examined in this figure were gated on 7-AAD negative cells to exclude dead cells. Data in this figure are representative of at least two independent experiments. Shaded histogram represents isotype control staining.

Sustained proliferation is necessary to maintain Rae1 expression

Once RAE-1 ε expression reached plateau levels on day 6-7, the expression was maintained indefinitely in the cultures when they were maintained in growth inducing conditions. To determine whether continued proliferation was necessary to maintain RAE-1 ε expression, 8 day cultured fibroblasts that expressed RAE-1 ε were exposed separately for 3 days to two anti-proliferative reagents, LY294002 (a PI3K inhibitor) or Roscovitine (a CDK inhibitor) (Fig. 3-8. *A*). Compared to control cells treated with DMSO, LY294002 and Roscovitine-treated fibroblasts exhibited a significant decrease in RAE-1 ε surface expression. RAE-1 ε expression was restored when the cells were washed to remove the reagents and cultured for 3 additional days (Fig. 3-8. *B*). Similarly, serum-starvation of primary cultured fibroblasts, which inhibits proliferation, resulted in a dramatic downregulation of RAE-1 ε surface expression (Fig. 3-9. *A*). Once proliferation was restored by the addition of serum to the cultures, RAE-1 ε expression was increased again to the same level as observed in control cells that had been maintained in serum throughout (Fig. 3-9. *B*). These data established that continuous proliferation is required for maintenance of cell surface RAE-1 ε expression.

Cell autonomous induction of RAE-1 ε *in proliferating cells*

Previously, it was shown that only 50~70% of mammalian cells re-enter the cell cycle after release from cell-cycle arrest (Bar-Joseph, et al., 2008). To test whether only proliferating cells induce RAE-1 ε, serum-starved primary cultured fibroblasts were stimulated with 10% serum in the presence of BrdU (Fig. 3-10). Compared to quiescent serum-starved cells (Fig. 3-10. *A*), more than 50% of serum-stimulated cells incorporated BrdU, indicating that they were replicating DNA (Fig. 3-10. *C*). As determined by gating on BrdU positive and negative populations, RAE-1 ε expression was observed only on BrdU positive cells (Fig. 3-10. *D*). Therefore, this data demonstrated that RAE-1 ε is induced in a cell autonomous manner in proliferating cells.

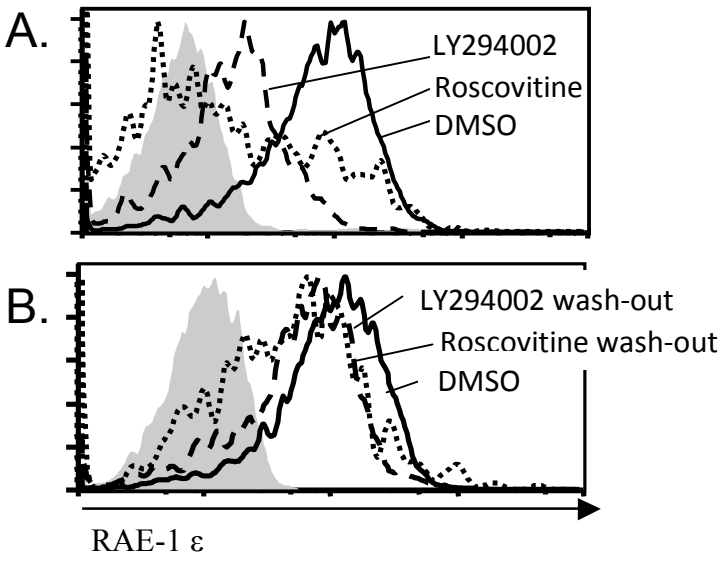

Fig. 3-8. Proliferation is required for maintenance of RAE-1 ε expression. A. Primary cultured fibroblasts that had been cultured for 8 days were treated with LY294002 or Roscovitine for 3 days. **B.** Cells from **A** were washed to remove the reagents and were cultured for 3 additional days. Data in this figure are representative of at least two independent experiments. All the cells examined in this figure were gated on 7-AAD negative cells to exclude dead cells. Shaded histogram represents isotype control staining.

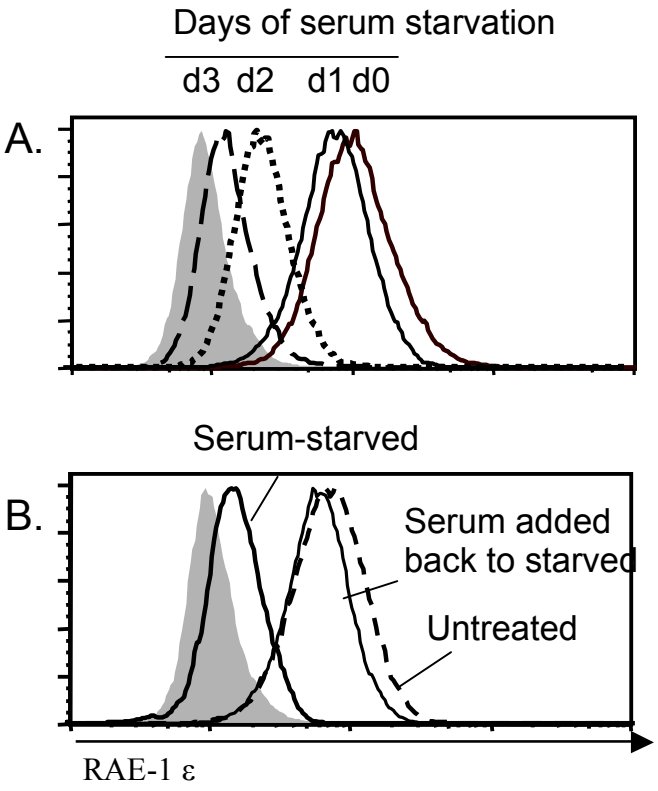

Fig. 3-9. Proliferation is required for maintenance of RAE-1 ε expression. Fibroblasts that had been cultured for months were serum-starved for 1~3 days (**A),** and then some of the 3 days serum-starved cells were supplemented with 10% serum and cultured for additional 3 days (**B**). Data in this figure are representative of five independent experiments. Shaded histogram represents isotype control staining.

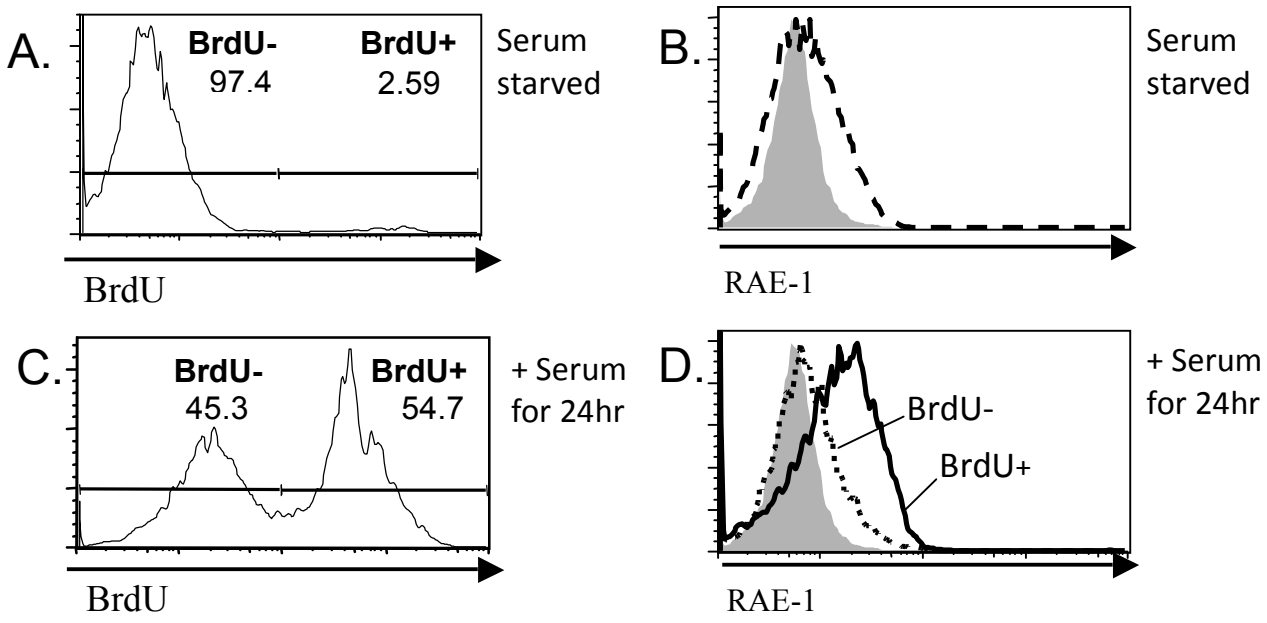

Fig. 3-10. Only proliferating cells induce RAE-1 ε expression. Primary cultured fibroblasts were serum-starved for 3 days. Some of the starved cells were supplemented with only BrdU (**A, B**) and the rest were supplemented with 10% serum and BrdU (**C, D**). Twenty four hours later, cells were harvested and stained with BrdU and RAE-1 ε antibodies. **D.** BrdU positive and negative populations gated from **C** are overlayed to show RAE-1 ε expression. All the cells examined in this figure were gated on 7-AAD negative cells to exclude dead cells. Data in this figure are representative of at least two independent experiments. Shaded histogram represents isotype control staining.

Susceptibility to NK killing

To address whether RAE-1 ε induction on proliferating cells was sufficient to induce activation of NK cells, proliferating RAE-1 ε + fibroblasts or fibroblasts that expressed low amounts of RAE-1 ε after 3 days of serum starvation were compared as target cells in a ^{51}Cr-release cytotoxicity assay, using IL-2 activated NK cells as effector cells (Fig. 3-11. *A*). Proliferating fibroblasts were lysed well by WT NK cells but much less well by NK cells from NKG2D-knockout mice, showing that most of the killing was NKG2D dependent. Serum-starved fibroblasts were killed significantly less well than proliferating fibroblasts, consistent with the lower amounts of RAE-1 ε on these cells. The modestly lower killing of the serum-starved fibroblasts by NKG2D-knockout NK cells suggested that the low amounts of RAE-1 ε remaining on these fibroblasts (Fig. 3-11 B) were sufficient to induce a low level killing. The fact that both proliferating and serum-starved fibroblasts were killed weakly but approximately equally by NKG2D-knockout NK cells suggests that the fibroblasts must express some other ligands that induce NK killing, and that expression of those ligands does not depend on cellular proliferation. These results demonstrated that RAE-1 ε induction induced by cellular proliferation is sufficient to induce cytolysis by NK cells in an NKG2D-dependent manner.

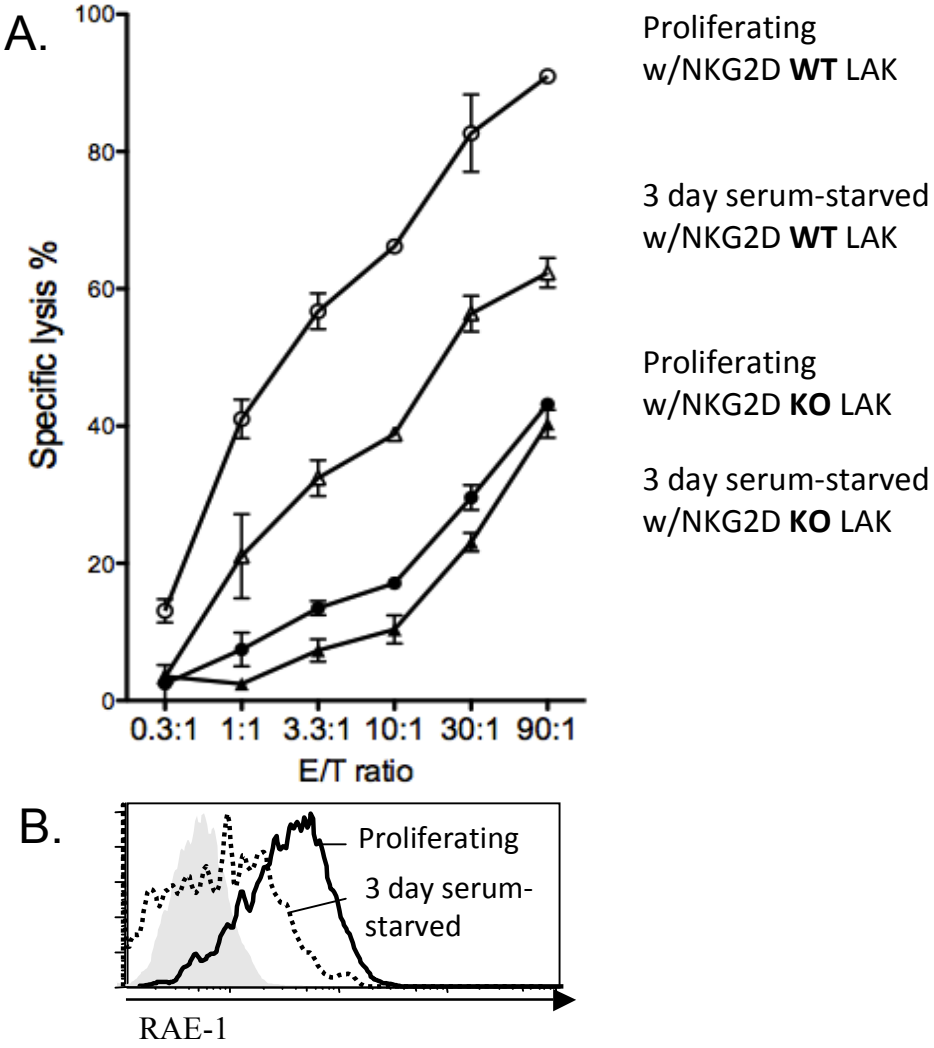

Fig. 3-11. Proliferating fibroblasts are more sensitive to NKG2D-dependent NK cell cytotoxicity. 5 day fibroblasts were serum-starved or not for 3 additional days. **A.** The fibroblasts were incubated with LAK cells prepared from NKG2D WT or NKG2D KO mice for 4 hours in the presence of ^{51}Cr and the radioactivity in the supernatant was measured in a Micro-beta counter. The spontaneous release was less than 17% of maximum release. **B.** Fibroblasts harvested before the cytotoxic assay were stained for RAE-1 ε, gating on 7-AAD negative cells to exclude dead cells. Data in this figure are representative of at least two independent experiments.

Expression in proliferating fetal brain cells in vivo

RAE-1 ε expression on tissues in normal healthy adult mice in vivo is thought to be very restricted. In contrast, significant expression of *Raet1* mRNA was detected in embryos at 11- 14- days of gestation, notably in the head region. By 18 days of gestation, Rae1 expression was beneath detection in the assays used (Zou, et al., 1996). Fetal brain cells are known to proliferate at a higher rate early in development (Daston, et al., 2004). These considerations raise several questions, including whether RAE-1 ε is expressed on the surface of fetal brain cells, whether the expression is dependent on stage of fetal development, and whether it correlates with the rates of proliferation of the cells. To address these questions, a single cell suspension of fetal brain cells from 14- and 18- day embryos were prepared without enzymatic treatments and stained with RAE-1 ε antibody. Strikingly, significant RAE-1 ε expression was detected on the surface of 14-day but not 18-day fetal brain cells by flow cytometry (Fig. 3-12 A). In addition, immunofluourescence analysis of sections of 14-day fetal brain by immunofluorescence showed RAE-1 ε staining consistent with cell surface and cytoplasmic localization of the protein (Fig. 3-12 B). To assess the proliferation rate of 14-and 18- day fetal brain cells, BrdU was injected into the mother 16 hours prior to harvesting the embryos. Analysis of mean fluorescence intensity (MFI) of BrdU staining showed that 14-day fetal brain cells have a significantly higher proliferation rate than 18-day fetal brain cells, consistent with a relationship between proliferation *in vivo* and Rae1 expression (Fig. 3-12 C). Interestingly, many day 14 brain cells were BrdU-low after 16 hours of labeling (Fig. 3-12 C) despite the fact that ~all the cells were Rae1+. A likely explanation is that ~all the cells are proliferating, but in an asynchronous fashion. The fact that day 18 fetal brain cells show significant residual proliferation but no detectable RAE-1 ε expression suggests either that RAE-1 ε expression depends on a threshold rate of cell proliferation that day 18 fetal brain cells no longer exceed, or that RAE-1 ε expression is specifically repressed at the later developmental stages.

The susceptibility of fetal brain cells to NK-dependent cytotoxicity was tested in a preliminary experiment. Although the extent of cytotoxicity was low, 14 day fetal brain cells were susceptible to killing by IL-2 activated NK cells in NKG2D-dependent manner, whereas no killing was observed when 18-day fetal brain cells were used as target cells (Fig. 3-12. D and data not shown). Taken together, these data indicate that fetal brain cells at the highly proliferative early stage express RAE-1 ε on the cell surface in a manner that renders the cells sensitive to NK killing. These findings support the strong correlation between proliferation and RAE-1 ε expression and extend the results to an *in vivo* system.

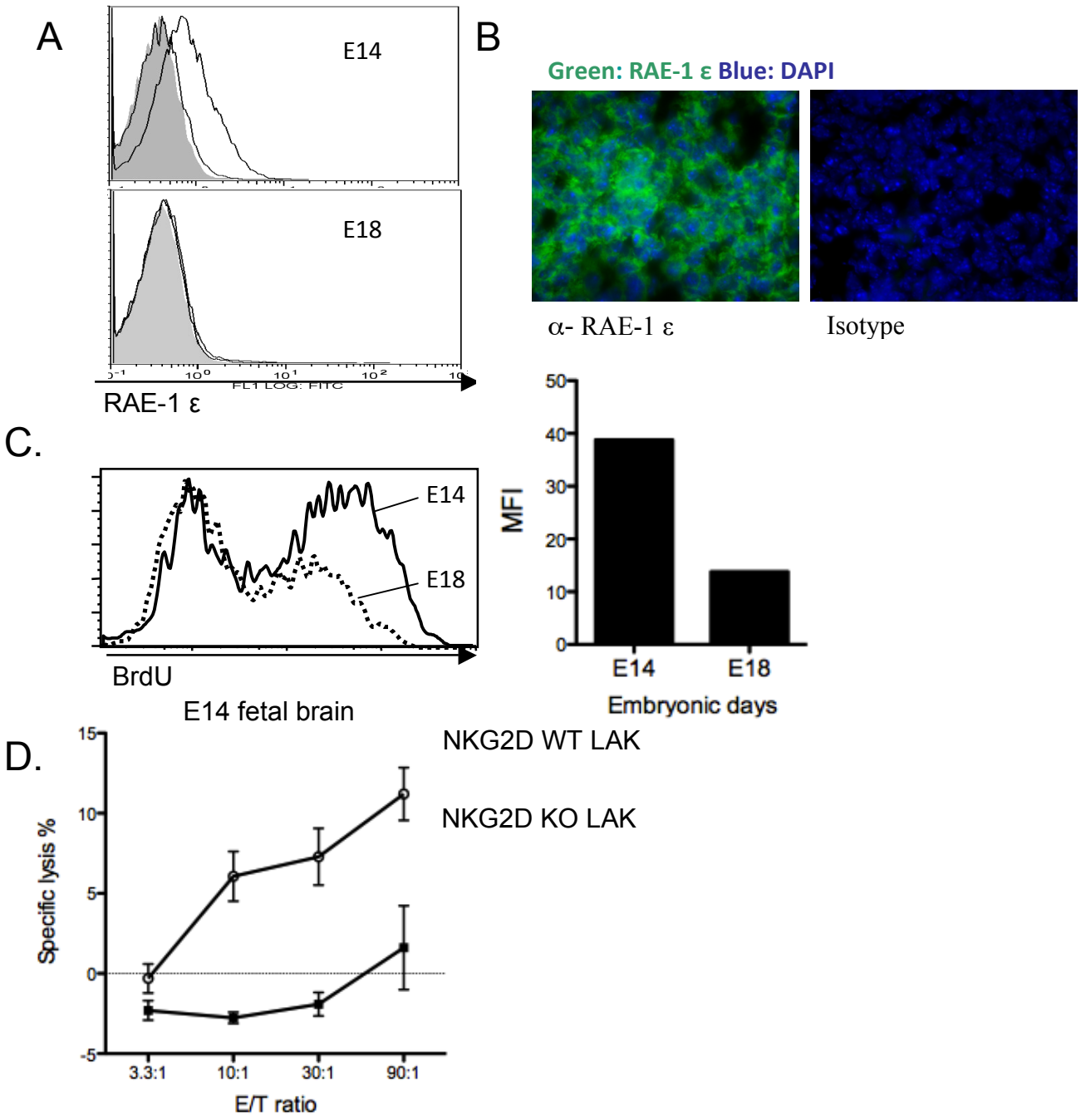

Fig. 3-12. Highly proliferative fetal brain cells induce RAE-1 ε on the surface *in vivo*. A. Single cells suspensions of embryonic day 14 and 18 fetal brains were stained with RAE-1 ε. **B.** Frozen sections (9 micron) of embryonic day 14 brain were stained with RAE-1 ε antibody (green) and DAPI (blue) **C.** 16 hours before sacrifice, pregnant mice were injected with BrdU (50mg/kg). Embryonic day 14, 18 fetal brains cells were harvested and stained with BrdU. MFI: Mean Fluorescence Intensity. D. Embryonic day 14 fetal brain cells were incubated with LAK cells prepared from NKG2D WT or NKG2D KO mice for 4 hours in the presence of ^{51}Cr and the radioactivity in the supernatant was measured in a Micro-beta counter. The spontaneous release was less than 16% of maximum release. Data in this figure are representative of at least two independent experiments. Shaded histogram represents isotype control staining.

Summary

Many tumor cell lines and primary tumors express NKG2D ligands, especially RAE-1 ε on the cell surface, but the mechanism of induction has not been clearly characterized. DNA damage, which is a well-known characteristic of tumor cells, has been shown to induce Rae1 in cultured cells and transformed cells (Gasser, et al., 2005) and heat shock response is also involved in cell surface protein expression of Mult1 (Nice, et al., 2009). In this chapter, I have found a new mechanism that regulates RAE-1 ε expression.

Upon primary culturing of fibroblasts, RAE-1 ε is induced without additional treatment. The induction was not dependent on DNA damage, oxidative stress or activation of tumor suppressor proteins, which are known to be induced in early culture. Fibroblast culture in a defined serum-free medium also sufficiently induced RAE-1 ε, however, RAE-1 ε induction was completed blocked when growth factor was not supplemented. This suggests that RAE-1 ε induction was dependent on proliferation. By inhibiting different pathways that induce proliferation or depriving serum from the culture condition, the role of proliferation was tested. RAE-1 ε induction was completely dependent on proliferation and sustained proliferation is required for maintaining expression. Also, only proliferating cells induce ligands, as opposed to the possibility that Rae1 is induced by global effect from growth factors.

RAE-1 ε induction on proliferating cells was sufficient to trigger NKG2D-dependent cytotoxicity of activated NK cells. Furthermore, RAE-1 ε is expressed on developing fetal brain cells at highly proliferative stage, which shows a link between proliferation and RAE-1 ε induction *in vivo*. Taken together, these data show that proliferation induces RAE-1 ε expression, which render cells sensitive to NK cell killing.

Chapter 4. Transcriptional regulation of *Raet1*ε in proliferating cells.

Raet1e mRNA induction

Previous studies of *Raet1e* regulation by the DNA damage stress response pathway showed that the increased expression at the cell surface was accompanied by increased amounts of *Raet1e* mRNA (Gasser, et al., 2005). To test whether *Raet1e* mRNA was also induced in association with the induction of proliferation in fibroblasts, the amounts of *Raet1e* transcripts were quantified by RT-qPCR in samples from tail tissue (d0) or after 2- or 10-days of culture in 10% serum (d2 or d10, respectively) (Fig. 4-1. *A*). We consistently observed >4~5 fold induction of *Raet1e* transcripts after 10 days in culture. Notably, no *Raet1e* mRNA was detected in d0 tail tissue, consistent with the hypothesis that most normal tissues do not express NKG2D ligands (Diefenbach, et al., 2000), (Zou, et al., 1996)). As mentioned in the preceding chapter, serum-starvation down regulates *Raet1e* cell surface expression. Analysis showed that starvation also resulted in a significant reduction in the amounts of *Raet1e* transcripts (at least 3-4 fold) within 24 hours of starvation and an even greater reduction thereafter (Fig. 4-1. *B*). These data demonstrated that *Raet1e* mRNA, like RAE-1 ε cell surface protein expression, is regulated by proliferative signals.

Transcription induction

Induction of *Raet1e* transcripts could be due to an increase in *Raet1e* transcription, or could reflect an increase in the stability of preexisting *Raet1e* transcripts. To address whether increased transcription occurs, nuclear run-on assays were performed to measure *de novo* transcription. Nuclei from proliferating or 3-day serum-starved fibroblasts were isolated and *in vitro* transcribed in the presence of Br-UTP for 15minutes, and the samples were then subjected to immunoprecipitation with antibodies specific for Br-UTP. The immunoprecipitated RNAs represent newly synthesized mRNA. The amounts of *Raet1e* transcripts in the newly transcribed RNA was determined by quantitative RT-PCR. Using primers that recognize unspliced *Raet1e* transcripts, we detected substantial amounts of newly synthesized *Raet1e* transcripts in the nuclei of proliferating fibroblasts and at least 4-5 fold less in serum-starved fibroblasts, indicating that transcription of the *Raet1e* gene is increased in proliferating cells (Fig. 4-2). As a control, a separate sample of proliferating fibroblasts was labeled in parallel with UTP instead of Br-UTP, and immunoprecipitated with Br-UTP-specific antibodies. As shown in . Fig. 4-2, basal amounts of *Raet1e* transcripts were immunoprecipitated under these conditions. These data indicate that cell proliferation results in increased transcription of the *Raet1e* gene.

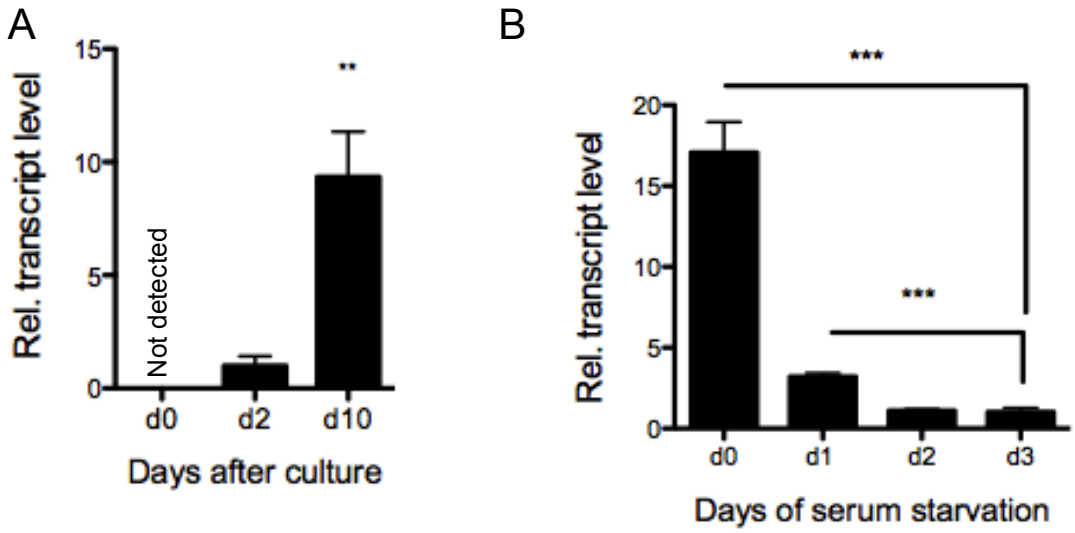

Fig. 4-1 *Raet1e* mRNA is induced in proliferating cells. Quantitative RT-PCR for *Raet1e* was performed on cDNA prepared from **A.** tail tissue (d0), 2 day or 10 day cultured fresh fibroblasts or **B.** fibroblasts starved for the indicated days. All transcript levels were normalized to 18s rRNA. Data are normalized to the day 2 amounts in panel A and to the day 3 amounts in panel B. Data in this figure are representative of at least five independent experiments. **=$p<0.005$, ***=$p<0.0005$ (Student's T-test)

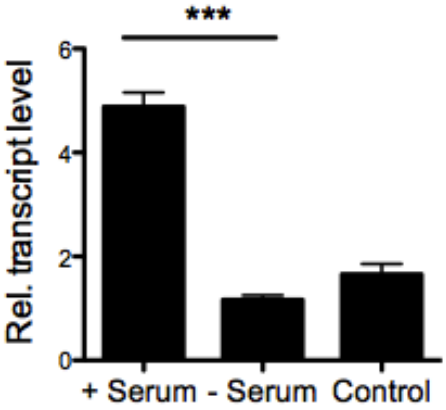

Fig. 4-2 *Raet1e* is transcriptionally induced in proliferating cells. Nuclear run-on assays were performed in the presence of Br-UTP in proliferating (+Serum), serum-starved (-Serum) fibroblasts or proliferating fibroblasts with UTP instead of Br-UTP (Control). Quantitative RT-PCR for Rae1 transcript was performed. All transcript levels were normalized to 18s rRNA. Data in this figure are representative of at least five independent experiments. ***=$p<0.0005$ (Student's T-test)

Promoter activity

The putative promoter region of the *Raet1e* gene was identified in a previous study performed in our lab, based on a combination of 5' RACE PCR, *in silico* promoter prediction analysis, and transactivation assays (Hsiung, Ph.D Thesis). To determine whether the promoter is more active in proliferating cells, a 274 bp fragment of the *Raet1e* promoter, which includes 205bp of the 5' flanking region and 69bp of the 1st exon, was cloned upstream of the luciferase gene in the pGL3-Basic vector, which lacks any other promoter or enhancer (Fig. 4-3 *A*). The data showed that the *Raet1e* promoter was active in the presence of serum, but was at least 3 fold less active in serum-starved fibroblasts (Fig. 4-3 *B*). A positive control reporter plasmid in which the luciferase gene was flanked by the SV40 promoter construct, was also active in fibroblasts cultured in serum, and also showed reduced activity in serum-starved cells. The reduced activity of the SV40 enhancer under conditions of serum-starvation was reported previously (Slansky, et al., 1993). These data suggest that the induction of Rae1 in proliferating cells is due to the increased activity of the Rae1 promoter under these conditions.

The E2F family of transcription factors may regulate the Rae1 gene

Among the many transcription factors that potentially regulate the Rae1 gene, we developed an interest in the E2F family of transcription factors due to their well established role in regulating proliferation. Three activating E2F family members are known (E2F1-3). As expected based on published literature, the amounts of transcripts of all three activating E2Fs decreased with kinetics similar to the reduction in *Raet1e* transcripts in serum-starved fibroblasts (Fig. 4-5. A). In addition, an analysis of the Gene Expression Omnibus website (URL http://www.ncbi.nlm.nih.gov/geo/) documented previous microarray data showing that Rae1 transcripts are significantly induced in cells transduced with an E2F1 expressing adenovirus (Ma, et al., 2002).

Also consistent with a role of E2F transcription factors were the results of an analysis of the region including the 5' UTR and the 1st exon with the Transcription Element Search System (TESS, URL: http://www.cbil.upenn.edu/tess). The E2F consensus sequence used to analyze the *Raet1e* promoter was TTTS(C/G)S(C/G)CGC, in which mismatches were allowed only in the T residues (Bieda, et al., 2006, Rabinovich, et al., 2008). The analysis showed that multiple putative E2F binding sites are localized in the putative promoter region, exon 1 and the first intron, whereas such sites were relatively rare in the downstream region of the gene (Fig. 4-4). It should be noted that recent genome-wide E2F1 ChIP analysis showed that most (82%) active E2F1 binding sites overlap with CpG islands, suggesting that the E2F sites found in the *Raet1e* CpG island that spans the promoter region and 1st exon could be the most important ones (Fig. 4-4) (Bieda, et al., 2006). We therefore examined the role of E2F transcription factors in greater detail. In addition, we examined the role of c-Myc,

because a previous study showed a correlation between c-Myc overexpression and *Raet1e* induction in lymphoma cells (Unni, 2008 (Unni, et al., 2008)).

E2F1 overexpression induces Rae1 mRNA

To test whether overexpression of E2F or c-Myc by transfection can induce *Raet1e* , fibroblasts were transduced with retroviruses expressing E2F1, E2F2, E2F3, or c-Myc, and RNA was harvested 48 hours later and analyzed by RT-qPCR. Strikingly, transfection of the E2F1 plasmid induced endogenous *Raet1e* expression by 4 fold. Transfection of E2F3 also modestly induced *Raet1e* transcripts, whereas E2F2 did not. Transfection of the Myc plasmid induced Rae1 modestly (and not always significantly statistically), but consistently (Fig. 4-5. *B,* data not shown). Analysis of transcripts of cyclin E, a known E2F target, showed significant induction by all the activating E2Fs as well as by c-Myc (Fig. 4-5. *C*).

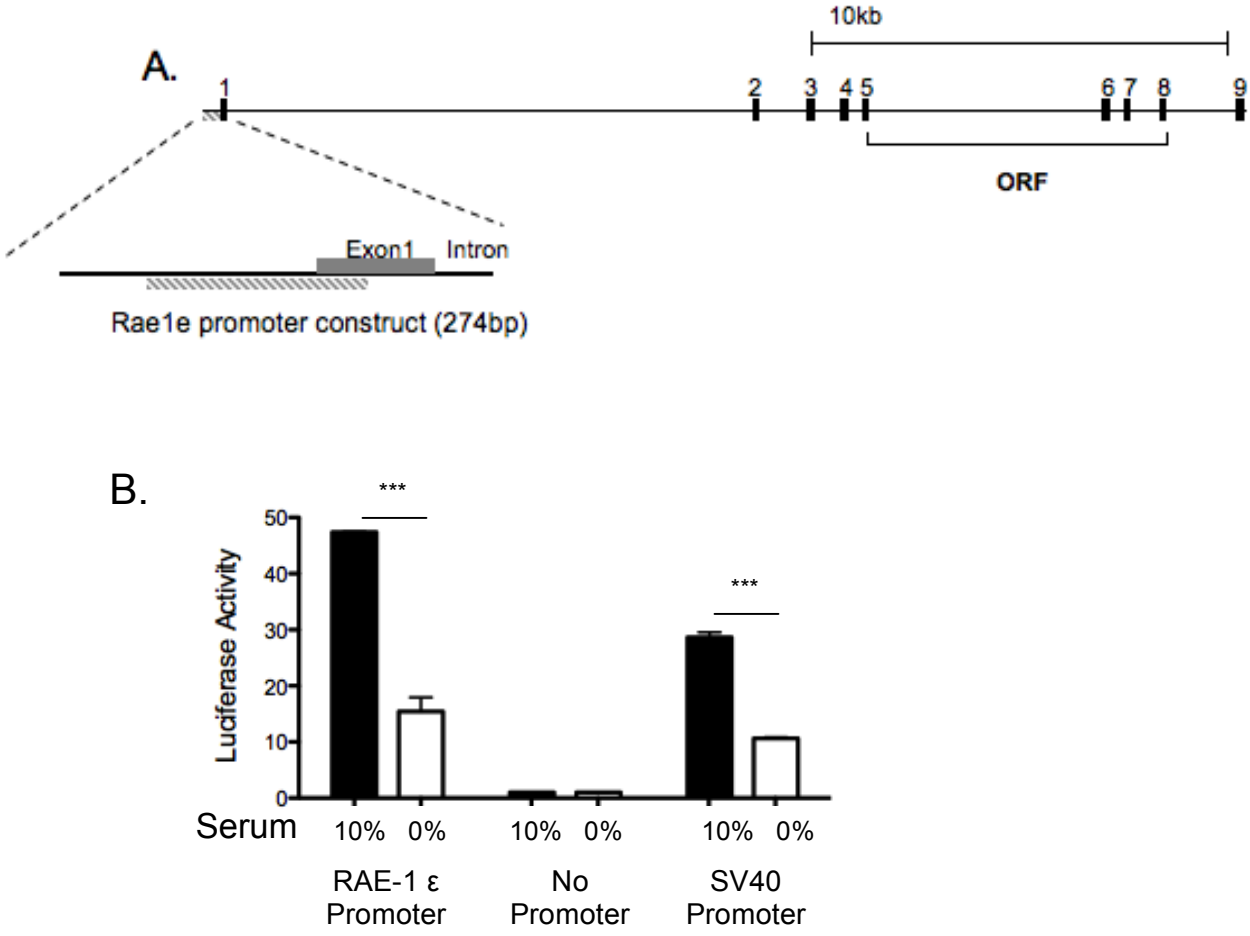

Fig. 4-3 *Raet1e* promoter is active in proliferating cells. A. A schematic of the *Raet1e* gene (drawn to scale). The promoter region is enlarged to show the location of the segment used for preparation of the *Raet1e* promoter reporter construct (hatched). **B.** Fibroblasts were transfected with pGL3 basic vectors containing the *Raet1e* promoter, no promoter or the SV40 promoter. The cells were incubated in 10% (black bar) or 0% (white bar) serum for 24 hours. In all cases a plasmid encoding Renilla luciferase driven by the SV40 promoter was cotransfected. The firefly luciferase activity in each sample was normalized to Renilla luciferase, and the values were normalized again to the no promoter control values. Data in this figure are representative of four or more independent experiments. ***=$p<0.0005$ (Student's T-test)

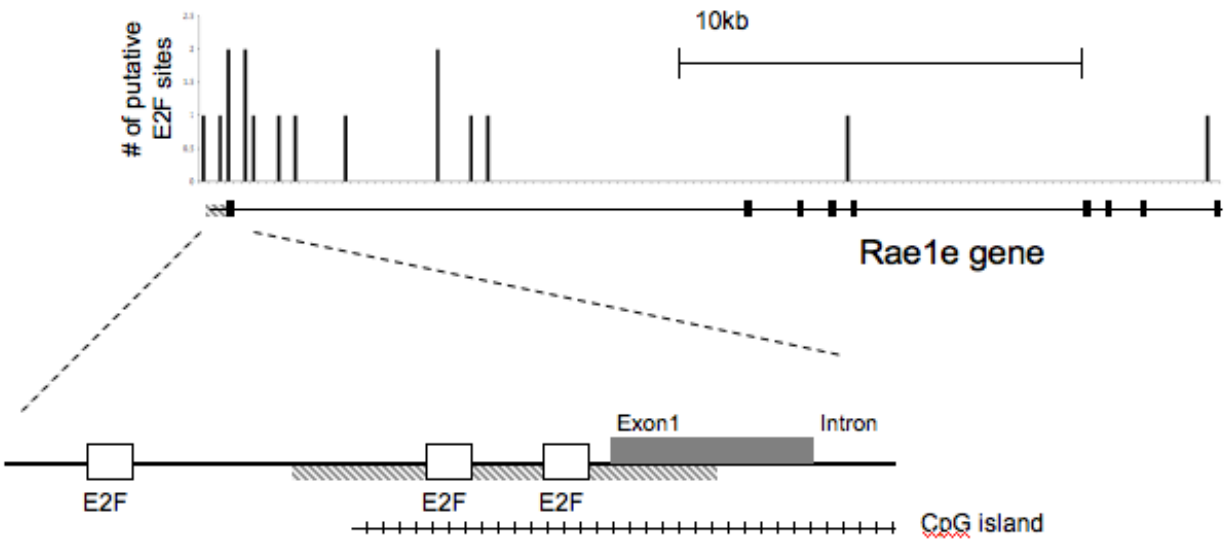

Fig. 4-4 Putative E2F binding sites in the *Raet1e* gene. Putative E2F binding sites were identified throughout the *Raet1e* gene using the TESS program, with the E2F consensus site defined as TTTSSCGC (S=C or G) with up to two mismatches allowed in T residues (drawn to scale). The 5' end of the gene is enlarged to show the locations of the segment used for preparation of the promoter construct, the CpG island and individual putative E2F sites. The hatched region shows the location of Rae1 promoter reporter construct.

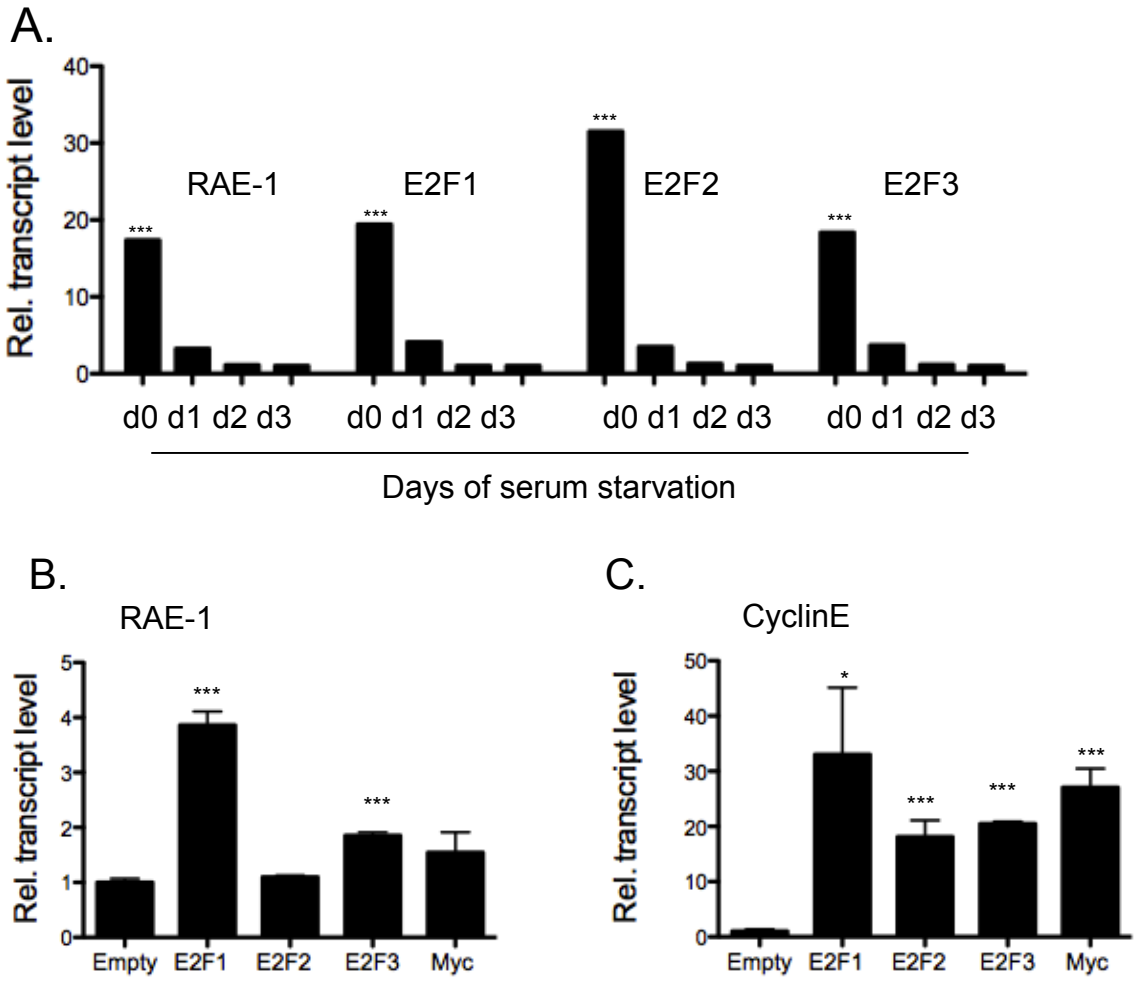

Fig. 4-5 E2F1 induces *Raet1e* mRNA. A.B. Fibroblasts were transduced with retrovirus expressing E2F1, E2F2, E2F3, c-Myc or GFP only and cultured for 48 hours in the absence of serum. *Raet1e* (**A**) and Cyclin E (**B**) transcripts were analyzed by quantitative RT-PCR. **C**. Fibroblasts were serum-starved for the indicated number of days and the RNA was analyzed for *Raet1e* , E2F1, E2F2 and E2F3 transcripts by quantitative RT-PCR. All transcript amounts were normalized to 18s rRNA. Data in this figure are representative of at least two independent experiments. Statistical analysis compared samples to the control samples transfected with Empty (GFP-only) vector (**A,B**) or d3-starved cells (**C**) *=$p<0.05$, ***=$p<0.0005$ (Student's T-test)

Chromatin Immunoprecipitation

In silico analysis and RNA analysis suggested the role of E2F transcription factors in regulating *Raet1e* . To assess whether E2Fs interact with the Rae1 gene in vivo, we performed chromatin immunoprecipitation (ChIP) assays. Nuclear extracts from proliferating or serum-starved (3 days) fibroblasts were sonicated (sheared 200 to 600bp) and immunoprecipiated with E2F1, E2F2 or E2F3 antibodies. The immunoprecipitated DNA was analyzed by semi-quantitative PCR for the amounts of sequences corresponding to the promoter region of Rae1, or that of p107, a known target gene for all three activating E2Fs (Iaquinta, et al., 2005). The primers were designed to amplify from -147 to +18 of the *Raet1e* gene, spanning the CpG island and including the tandem E2F sites. As shown in Fig. 4-6, E2F1 was bound to the *Raet1e* promoter region in proliferating cells. The amount bound was reduced ~4-fold in serum-starved cells, considering the difference in the input amounts. Preliminary results also indicated that E2F2, but not E2F3 was bound to the *Raet1e* promoter region in proliferating cells, with no binding detected in serum-starved cells. In contrast, E2F1, E2F2 and E2F3 all bound to the p107 promoter region, demonstrating that the assay worked efficiently. The finding that E2F2, but not E2F3 bound to the *Raet1e* promoter was surprising, considering that transfection of E2F2 had no effect whereas E2F3 transfection consistently resulted in modest induction of *Raet1e* RNA. It remains to be seen whether the results with E2F2 and E2F3 are reproducible as it was done only once.

Taken together, these data showed that E2F1, at least, binds to the promoter region of *Raet1e* in proliferating fibroblasts. Although ChIP results alone do not demonstrate transcriptional regulation by a factor, the ChIP results, combined with the induction of *Raet1e* transcripts in cells transfected with E2F1 (Fig. 4-5), argue that E2F1 can induce *Raet1e* transcription by binding to the promoter region.

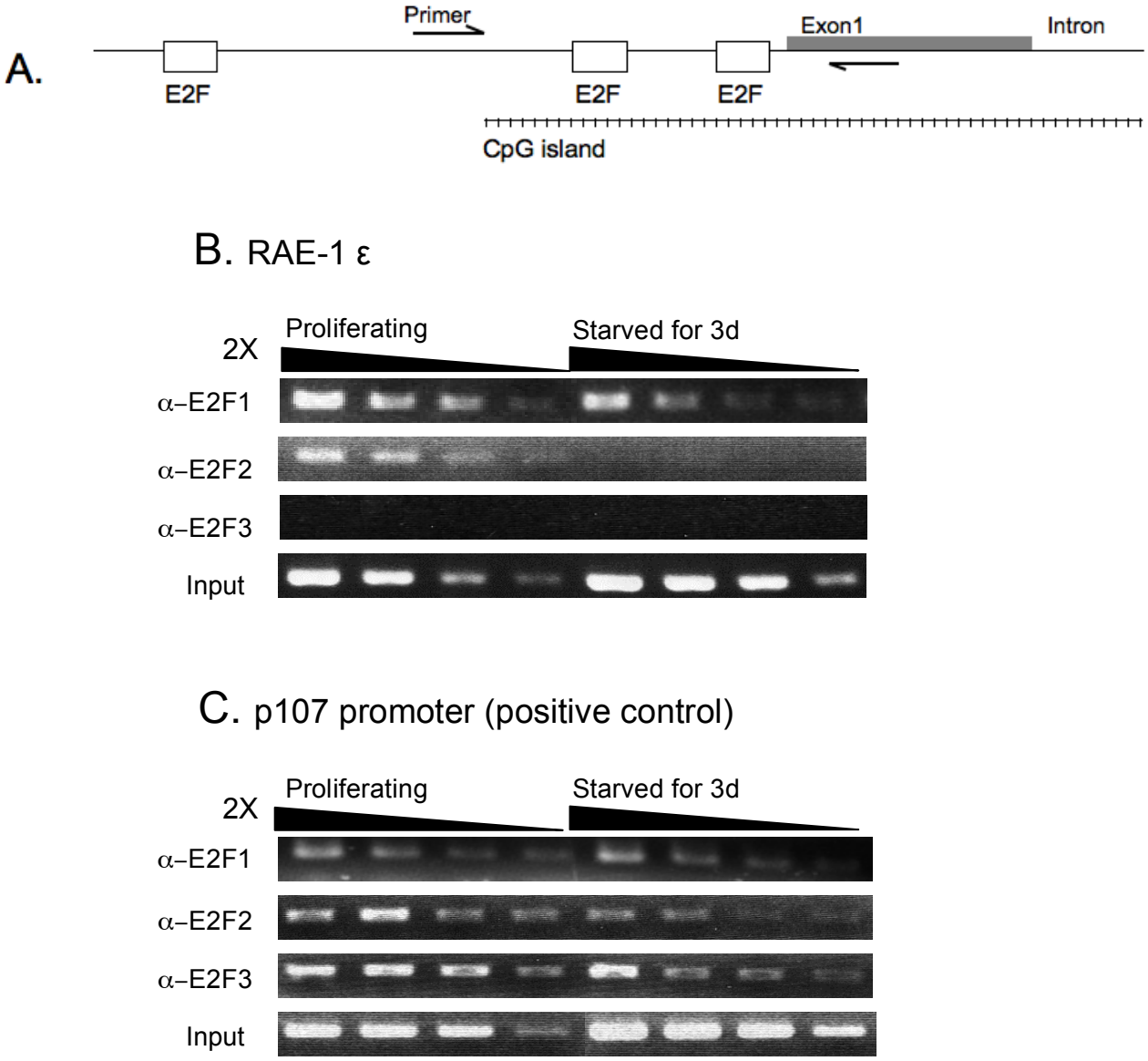

Fig. 4-6 Chromatin precipitation assay of E2F binding to the *Raet1e* promoter. A. Schematic of the *Raet1e* gene depicting putative E2F binding sites in the promoter regions and exon1. **B.** E2F1 and possibly E2F2 binds to the *Raet1e* promoter in proliferating cells. ChIP was performed using antibodies against E2F1, E2F2,or E2F3, or using an IgG isotype control. PCR primers for the *Raet1e* promoter were designed to yield a 165 bp product spanning from -147 to +18 (panel A). The input lane represents 0.3% of the amount of chromatin used in the ChIP assays. **C.** As a positive control, primers that recognize the promoter of a common target gene, p107, were used. E2F1 ChIP is representative of 4 independent experiments. E2F2 and E2F3 ChIP was performed once.

E2F1 directly transactivates the Raet1e promoter

To directly investigate whether E2F1 regulates the *Raet1e* promoter, the reporter constructs described in Fig.4-3 were transfected into fibroblasts with pcDNA vectors encoding E2F1, E2F2 or c-Myc, or empty pcDNA vector, and the luciferase activity was measured. E2F1, E2F2 and c-Myc plasmids all transactivated the positive control SV40 promoter construct, consistent with evidence that the SV40 promoter is induced 2-3 fold by serum stimulation (Slansky, et al., 1993). E2F1 strongly transactivated the *Raet1e* promoter, supporting a role for E2F1 in directly regulating the *Raet1e* gene. A separate experiment in which the amount of E2F1 vector was varied showed a dose dependent induction of the *Raet1e* promoter by E2F1, to levels higher than were observed in cells stimulated with 10% serum (Fig. 4-8). In contrast, transfection of the E2F2 vector did not induce *Raet1e* promoter activity (Fig. 4-7).

The c-Myc plasmid modestly induced the *Raet1e* promoter as well (Fig. 4-7). However, the activity was not augmented by increasing the dose of c-Myc plasmid. These data suggest that Myc may act indirectly, possibly by inducing proliferation or increasing the availability of E2F1. Note that both E2F1 and Myc overexpression both potently induce cell cycle entry and to a similar amount. The fact that E2F1 potently induces the *Raet1e* promoter and c-Myc does so only modestly suggests that the effect of E2F is unlikely to be due simply to the induction of cell cycle entry.

It remained possible that E2F1 acts indirectly in the induction of the *Raet1e* promoter. To test whether the E2F1 sites in the *Raet1e* promoter are important for transactivation, mutations were introduced into each of the two putative E2F binding sites identified with the TESS program. The first E2F consensus site -56/TTCCCCGC was mutated to TTCATATGC, and the second consensus site -10/TGCCCCGC was mutated to TGCATATGC. Mutation of each site separately caused a significant reduction in promoter activity. Mutation of both simultaneously caused an even greater reduction in activity, though some activity remained in some experiments, possibly due to other, cryptic, E2F sites in this region (see discussion) (Fig. 4-9). These data show that E2F1 regulates the *Raet1e* promoter through direct binding.

A previous report concluded that c-Myc regulates the Rae1 gene, based on the finding that *Raet1e* transcripts were induced B cell lymphomas arising in Eu-Myc transgenic mice, and that c-Myc binds to a site in exon1 of *Raet1e* gene (Unni, et al., 2008). Because E2Fs have been shown to cooperate with myc in transcription of some target genes (Leung, et al., 2008, Leone, et al., 2001), I tested whether c-myc synergizes E2F1 in the induction of *Raet1e* transcripts in transfected cells or in the transactivation of the Rae1 promoter. Preliminary result showed c-Myc overexpression inconsistently induced *Raet1e* mRNA up to 2 fold and only modestly induced the *Raet1e* promoter (~2 fold) (Fig. 4-7). A preliminary experiment showed that c-Myc and E2F1 together transactivated the *Raet1e* promoter better than either alone, but in an additive, rather

than synergistic, fashion. Further experiments will be required to determine the role of c-Myc in the induction of the Rae1 gene (see Discussion).

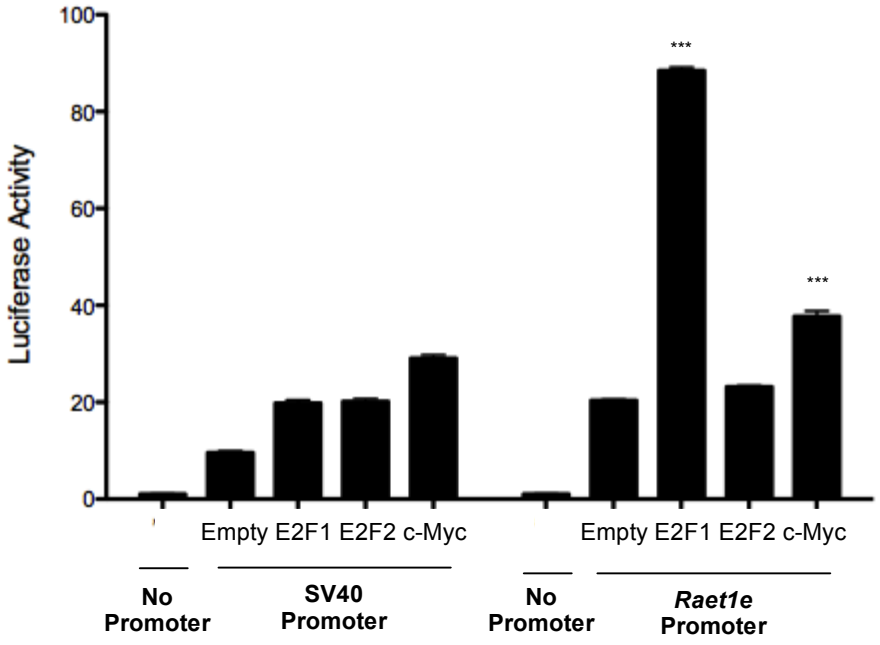

Fig. 4-7. E2F1 but not E2F2 transactivates the Rae1 promoter reporter construct. Fibroblasts were transfected with 50 ng pGL3 vector containing the SV40 promoter, the *Raet1e* promoter or no promoter, along with 750 ng of empty pcDNA vector or pcDNA encoding E2F1, E2F2 or c-Myc. In all cases a plasmid encoding Renilla luciferase driven by the SV40 promoter was cotransfected. The firefly luciferase activity in each sample was normalized to Renilla luciferase, and the values were normalized again to the no promoter control values. Data in this figure are representative of three independent experiments. The statistical analysis compared samples to the empty vector control ***=$p<0.0005$ (Student's T-test)

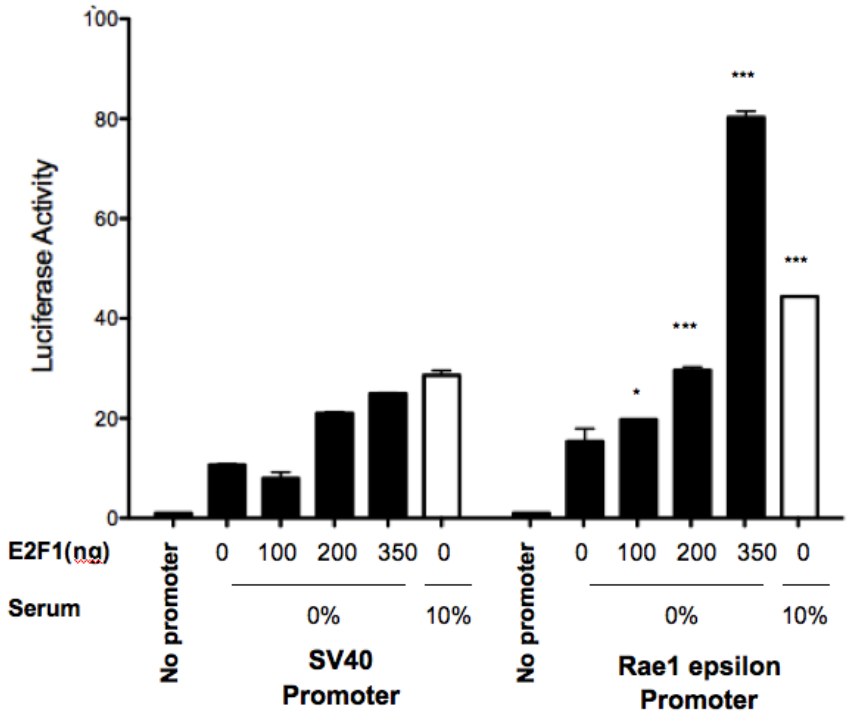

Fig. 4-8. E2F1 transactivates Rae1 promoter in dose-dependent manner. Fibroblasts were transfected with 50ng pGL3 vector containing the SV40 promoter, the *Raet1e* promoter or no promoter, along with varying amounts of pcDNA encoding E2F1 (0~350ng). The total mass of transfected DNA in each well was kept constant by adding empty pcDNA vector (up to 800ng). The samples were normalized to the cotransfected Renilla luciferase values as before, and normalized again to the no promoter control. Data in this figure are representative of three independent experiments. The statistical analysis compared samples to the empty vector control. *= $p<0.005$, ***=$p<0.0005$ (Student's T-test)

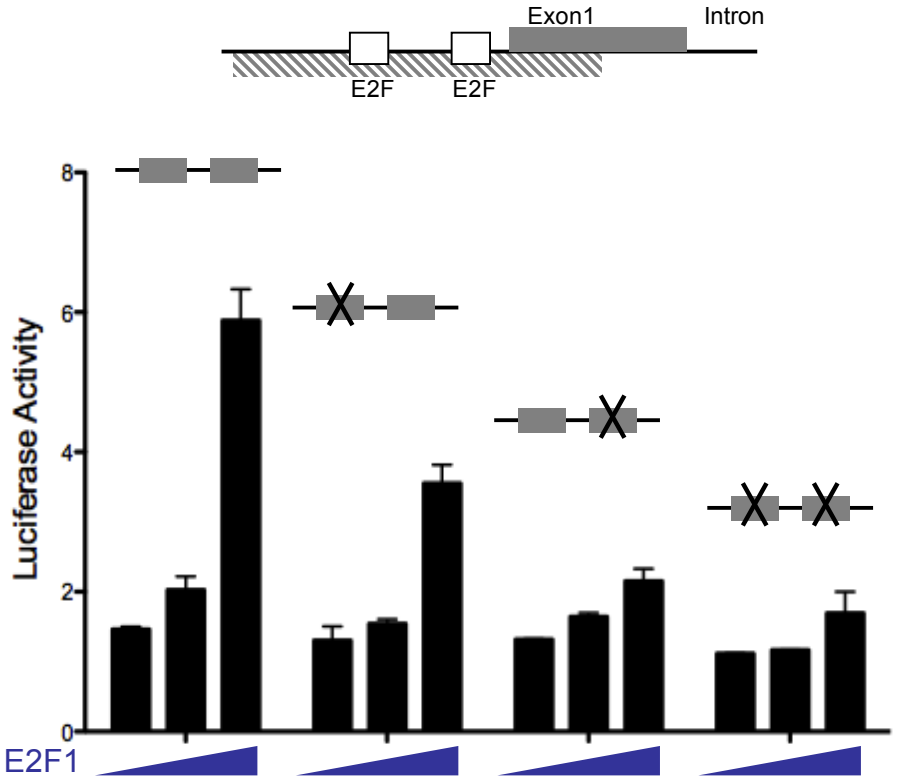

Fig. 4-9. E2F1 directly transactivates the Rae1 promoter construct. Fibroblasts were transfected with 50 ng pGL3 vector containing the *Raet1e* promoter segment or corresponding segments in which each putative E2F site was mutated or both were mutated, along with varying amount pcDNA encoding E2F1 (0-350ng). The total mass of transfected DNA in each well was kept constant by adding empty pcDNA vector (up to 800ng). Data were normalized as in Fig. 4-7. Data in this figure are representative of four independent experiments.

Analysis of 5' flanking region of other NKG2D ligands

To investigate whether other NKG2D ligands are regulated by similar mechanism, 5' flanking region of known NKG2D ligands were analyzed for homology with the promoter region of *Raet1e* and putative E2F binding sites.

Alignment of human NKG2D ligands and 274bp promoter/exon1 sequence of *Raet1e* gave no significant homology. Surprisingly, however, I have found that 5' flanking region and exon1 of H60b, one of mouse ligand, matched ~95% with the Rae1 promoter/exon1 sequence. The first exon of H60b exactly matched with part of *Raet1e* exon1 and 167bp of H60b 5' flanking region showed 94% identity with *Raet1e* sequence which covers part of exon1 and promoter regions (Fig. 4-10 *A*). H60b is relatively recently discovered NKG2D ligands (Takada et al., 2008), and its regulation is not yet known. This analysis suggests that H60b could potentially be regulated similarly by proliferative signal, through E2F1.

In searching for homologous sequence throughout mouse genome using UCSC genome browser (URL: http://genome.ucsc.edu/), I found unexpected two regions with ~90% identity. First region is at ~5kb upstream from *Raet1e* transcription start site in chromosome 10 (3341207~3341534), with 91.8% identity and the second region is at ~2kb upstream from Cdk6 transcription start site in chromosome 5 (3341207~3341534), with 88.9% identity. Both regions located in CpG island. The significance of the regions are unclear, but potentially important as a regulatory element.

Although there were not significant homologies between 5' flanking region of human NKG2D ligands and *Raet1e*, analysis for putative E2F sites showed that there are multiple potential binding sites for E2Fs in 5' flanking region of most human NKG2D ligands, except ULBP4 (RAET1E) (Fig. 4-10 *B*). Furthermore, all of the 5' flanking region of human NKG2D ligands, except ULBP4 (RAET1E), located in CpG island, which strongly suggests that the sites might be *bona fide* E2F binding sites (Bieda et al,. 2006).

Further studies on the transcriptional regulation of human NKG2D ligands or H60b by proliferation should provide important insights on the role of proliferative signals in alerting immune cells through NKG2D receptor-ligand interaction and potentially extend the results in human system.

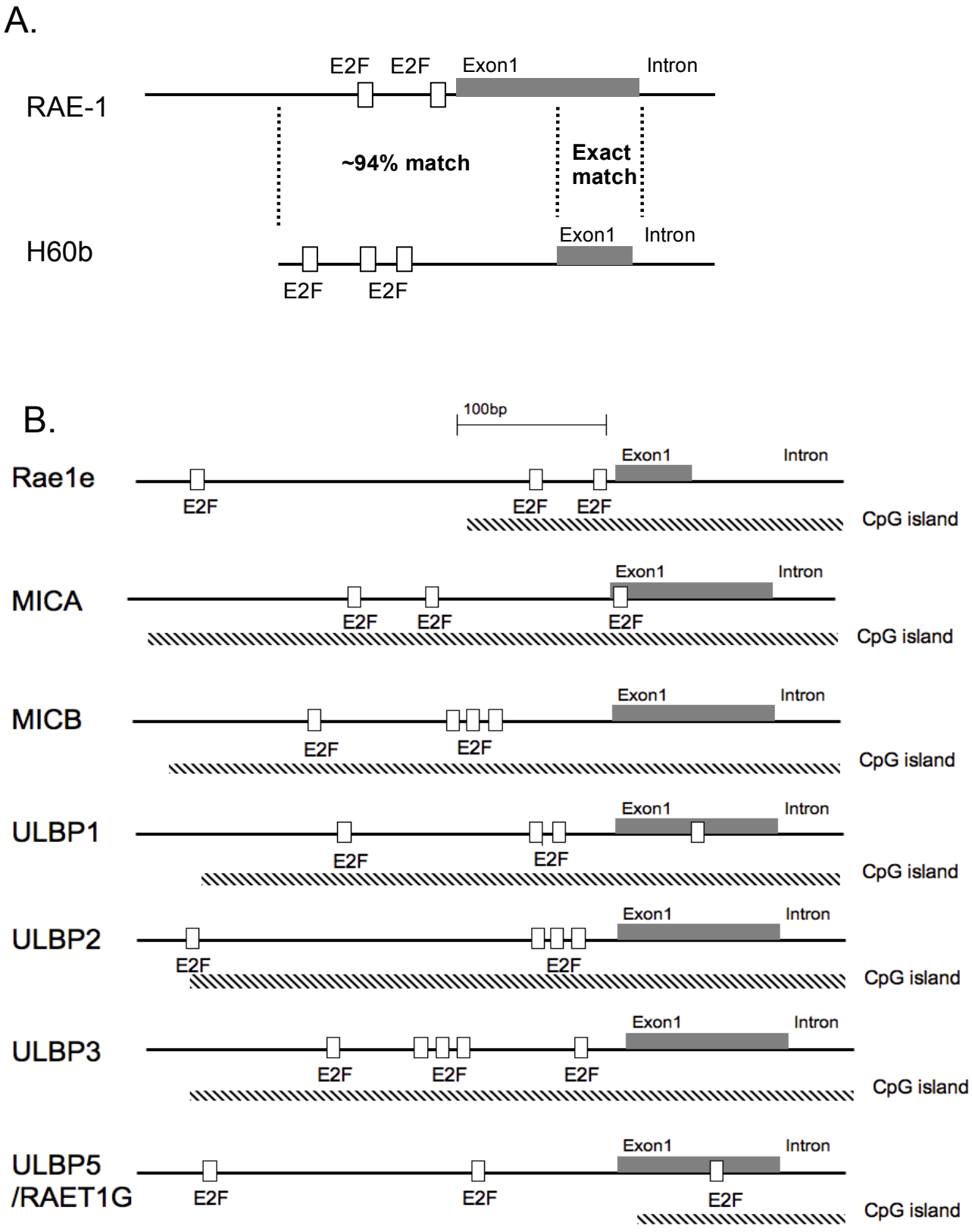
A.
RAE-1
E2F
E2F
Exon1
Intron
~94% match
Exact match
H60b
Exon1
Intron
E2F
E2F
B.
100bp
Rae1e
MICA
MICB
ULBP1
ULBP2
ULBP3
ULBP5 /RAET1G
Exon1
Intron
E2F
CpG island

Fig. 4-10. *In silico* analysis of 5' flanking regions in various NKG2D ligands. A. Schematics of 5' flanking regions and exon1 of *Raet1e* and H60b, depicting putative E2F binding sites. Dotted line shows regions of sequence matched *Raet1e* and H60b gene with different identity (94% identity and exact match). B. Schematics of 5' flanking regions and exon1 of human NKG2D ligands, depicting putative E2F sites (TTTC/GC/GCGC with up to two mismatches in T) and CpG islands (hatched).

Summary

NKG2D is a potent activating receptor expressed by various immune cells. Regulation of NKG2D ligands plays an important role in determining whether NK cells or other effector cells destroy the cells. Evidence suggests regulation of NKG2D ligands at several levels of biogenesis, but little is known concnerning how they are regulated at the transcriptional level. In this chapter, I described a novel mechanism for regulating expression of *Raet1e* at the transcriptional level. *Raet1e* mRNA is absent in most normal cells, but *Raet1e* mRNA was significantly increased when normal fibroblasts were induced to proliferate by culturing the cells in serum growth factors in culture. Analysis in this chapter showed that *Raet1e* transcription, as opposed to mRNA stabilization, was induced in proliferating cells. With the use of reporter plasmids, it was shown that the *Raet1e* promoter is more active in proliferating cells than in serum-starved cells. Furthermore, overexpression of E2F1 induced *Raet1e* mRNA in serum-starved cells. Finally, E2F1 transactivated the *Raet1e* promoter reporter plasmid, and mutation of potential binding sites in the promoter segment resulted in substantial reductions in the sensitivity of the promoter to E2F1 transactivation. Taken together, these data show that E2F1, which is upregulated in proliferating cells, induces transcription of Rae1.

Chapter 5. Discussion

Transcriptional induction of Rae1 in response to a signal associated with proliferation, via the action of the E2F1 transcription factor, represents a novel mechanism of regulation of NKG2D ligand genes. A published study reported that transcription of another NKG2D ligand gene, human MICA, is regulated by the heat shock pathway. The *Raet1e* gene is a member of a distinct family of NKG2D ligand genes (the Raet1 family) that also includes the human ULBP genes. There is no direct evidence that any member of this family of ligands is regulated transcriptionally by the heat shock stress pathway, though it has been reported that one such ligand, Mult1, is regulated post-translationally by heat shock associated signals. It was previously reported that c-Myc associates with the *Raet1e* gene, but no evidence was presented that c-Myc regulates transcription of the gene. Other studies showed that expression of Rae1 and ULBP family genes is regulated by the DNA damage response, but unpublished evidence showed that this regulation occurred post-transcriptionally, apparently by regulating the stability of the Rae1 mRNA. Hence the results presented in this thesis provide the first evidence to date concerning factors that directly regulate transcription of Raet1 family genes.

The demonstration that proliferation-related signals regulate the *Raet1e* gene is of particular interest because deregulated proliferation is a first sign of malignancy. This finding suggests one mechanism whereby properties of potentially malignant cells signal Rae1 expression in order to alert immune cells to the presence of transformed cells.

Totality of signals required for induction of Rae1 at the cell surface

Obviously, proliferation is not restricted to malignant cells, but also occurs during development of normal tissues and during processes of tissue renewal. The present data suggest that the expression of Rae1 should be assessed for various normal proliferating cell types. An analysis of cells that accumulated in healing skin wounds suggested that the proliferating non-hematopoietic cells in the wound did not express RAE-1 ε appreciably, suggesting that proliferation is not always sufficient to induce Rae1 (Appendix 1). Similarly, the data in Chapter 4 show that proliferating brain cells at day 18 of gestation do not express appreciable RAE-1 ε. In addition, preliminary analysis suggested that T cells proliferating during immune responses in vivo do not express high levels of Rae1 at the cell surface (Sophie Lehar and David Raulet, unpublished data). These results support the paradigm that Rae1 expression is generally restricted to tumor cells and other unhealthy cells. These considerations pose a conundrum as to why Rae1 cell surface expression is not induced in all proliferating cells.

One possibility is that Rae1 induction only occurs when the rate of proliferation exceeds a high threshold, which is not achieved during most normal processes. A higher threshold requirement of proliferation could in turn reflect a requirement for a relatively

high concentration of E2F1 to support *Raet1e* transcription. A requirement for a threshold amount of proliferation could explain why Rae1 is induced in brain cells at day 14 of gestation, but not at day 18, despite the fact that day 18 brain cells continue to proliferate, though at a slower rate than at day 14 (Fig.3-10). Also consistent with this possibility was the observation that much more E2F1 plasmid was apparently required for induction of Rae1 than for induction of other targets (a ratio of at least 5:1 of E2F1 to reporter construct for Rae1, compared to ratios of 1:1, 0.03:1 or 0.002:1 for p73, cyclin D3 and cyclin E, respectively, Fig. 4-8, (Ohtani, et al., 1995, Stiewe and Putzer, 2000, Ma, et al., 2003). These possibilities could be explored further by stimulating the cells with increasing doses of growth factors and comparing the amounts of E2F1 in the cells, the occupancy of these genes by E2F1, and the extent of gene expression.

Although a threshold proliferation requirement for Rae1 induction may account for some instances where proliferating normal cells fail to induce Rae1 expression, other cases are likely to require a different explanation. For example, T cells proliferate at a very high rate during immune responses, doubling at a rate ~4 times a day, yet do not induce high levels of cell surface Rae1.

One possibility is that some cell types that proliferate rapidly in vivo, such as T lymphocytes, or brain cells at later stages of development, incorporate mechanisms to specifically block Rae1 expression, in order to prevent elimination of useful immune responses. One finding that may be consistent with this proposal is that Rae1 expression was consistently absent on T lymphomas that developed in p53 knockout mice, even when the tumors arose in NKG2D KO mice and hence could not have been selected for loss of NKG2D ligands (Nadia Guerra, and D. Raulet, unpublished data).

Another model is that whereas proliferation is important for inducing *Raet1e* transcription, it is not sufficient for inducing Rae1 at the cell surface. In fact, other evidence supports the idea that the different stages of biogenesis of NKG2D ligands are regulated by different mechanisms. For example, the DNA damage response was shown to augment the stability of *Raet1e* transcripts (Hsiung, B, PhD thesis), whereas heat shock induced the Mult1 protein by decreasing the rate of ubiquitin dependent Mult1 turnover (Nice, et al., 2009). Another study demonstrated that PI-3 kinase activation is necessary for maximal expression of Rae1 in virus infected cells and cell lines, and suggested that this may occur in part at the level of translation (Tokuyama, et al, submitted for publication). It was proposed that optimal induction of NKG2D ligands involves the synergistic effects of multiple stress pathways acting together to support ligand gene transcription, mRNA stability, protein stability etc (Raulet and Guerra, 2009). These stress pathways, and proliferation, are often concomitantly induced in tumorigenesis and other disease processes, but are rarely concomitantly activated in healthy cells. In the case of healthy proliferating cells, for example, Rae1 transcription may occur but the other stress pathways required may not act to stabilize the mRNA, support maximal translation, or stabilize the Rae1 protein, etc.

The hypothesis that signals associated with several features of cancer cells synergistically induce expression of Rae1 and other NKG2D ligands is attractive

because it provides an explanation for how normality is distinguished from the cancerous state. However, my data may raise some doubts concerning the validity of this model. I demonstrated that normal fibroblasts placed in culture with growth factors induced cell surface Rae1 expression. Furthermore, I showed that inhibition of the ATM-ATR dependent DNA-damage response (DDR) pathway, which is active in proliferating fibroblast (Fig. 3-2A) did not inhibit Rae1 induction (Fig. 3-2B)..These findings might seem contradictory to the hypothesis that multiple pathways, including the DDR, are necessary for optimally inducing NKG2D ligands. However, these findings do not rule out the hypothesis.

One possibility is that the large amounts of growth signals provided in culture stimulate enough Rae1 transcription to overcome the requirement for the DDR to stabilize the Rae1 mRNA. This could be tested by examining Rae1 induction in cells with limiting amounts of growth factors.

Another, interesting, possibility is that the DDR has opposing effects in normal cells that mask its role in the induction of Rae1 in my experiments. This makes sense because the DDR is known to cause cell cycle arrest and thereby block cell proliferation, which is predicted to inhibit Rae1 gene transcription, whereas other evidence suggests that it stabilizes Rae1 mRNA, which should increase Rae1 expression. These two opposing effects may cancel out in my experiments, leading to no marked change in Rae1 expression. Consistent with this possibility, DNA damaging agents were the only inhibitors of proliferation that I tested that did not inhibit Rae1 expression—instead they either had no effect or slightly induced Rae1 expression, especially when limiting amounts of aphidicolin was applied (Fig. 3-6 and preliminary results).

This proposal can be tested in additional experiments in which the rate of Rae1 transcription an/or the stability of Rae1 mRNA is assessed in cultured fibroblasts treated or not with inhibitors of the DDR or with DNA damaging agents. Other potentially informative experiments would test whether Rae1 expression is reduced by inhibiting ATR and ATM in proliferating p53-/- fibroblasts, which largely fail to arrest in response to an active DDR. A similar experiment would test whether Rae1 expression is induced by DNA damaging agents in proliferating p53-/- fibroblasts. p53 is presumably unnecessary for stabilizing Rae1 mRNA, based on the evidence that DNA damage induced Rae1 expression in p53-/- cell lines.

Another consideration relevant to this discussion is that growth arrest induced by the DDR, unlike growth arrest induced by the other inhibitors used, occurs predominantly in S-phase, during which E2F1 is normally highly expressed. Therefore, it is possible that the active DDR results in arrest of cells in conditions where transcription can still occur and this could account for the failure of DNA damaging drugs to inhibit Rae1 transcription. It would be of interest to test whether Rae1 transcription, like that of many E2F1 targets, cycles throughout the cell cycle, increasing in late G1 and S phase and decreasing thereafter.

Expression of Rae1 in Proliferating Normal Cell Types

As mentioned earlier, cell proliferation frequently occurs in healthy tissue *in vivo*. One example is developing tissue. I have shown that rapidly proliferating fetal brain cells express Rae1 on the surface, which renders the cells sensitive to NK killing in vitro (Fig.3-12). It is important to empahsize that at day 14 of gestation, there are no mature NK cells or T cells, so the expression of Rae1 in the early fetal brain may be without immunological consequence. Rae1 expression disappears from mouse brain cells by day 18 of gestation, well before mature NK cells appear in mice. It remains of interest whether Rae1 appears on any normal proliferating cells at later stages of development, when functional NK cells are present in significant numbers (after birth in mice).

Gut epithelium is an interesting tissue to examine, as precursor cells proliferate frequently to replace dying cells. In human, MICA protein is reportedly expressed in the gut epithelium, although the mechanism of MICA induction itself remains unclear (Groh, et al., 1996, Hue, et al., 2004). It would be of interest to see whether Rae1 is induced in mouse gut epithelium, and whether it is associated with cell proliferation.

Another well-known site of proliferation *in vivo* is in healing wounds (Appendix 1). During the wound healing process, keratinocytes and fibroblasts proliferate locally and remodel the damaged tissue. Although we have not obtained clear evidence for expression of Rae1 protein on the surface of cells in healing wounds, I did observe that wound healing in NKG2D deficient mice was significantly delayed compared to wound healing in WT mice, suggesting that NKG2D receptor- ligand interactions plays a role in this process (Fig. A1-1). Moreover, I observed that Rae1 mRNA is induced significantly in wounded tissue. Unexpectedly, however, I found that Rae1 mRNA was induced in the CD45+ (hematopoietic) population. However, I failed to detect high Rae1 transcript levels in the CD45- (nonhematopoietic) population of cells in wounded tissue, which are known to include many proliferating cells . This finding seems to argue that proliferating fibroblasts and keratinocytes *in vivo* may not induce Rae1 RNA. However, we recently determined that although BrdU+ (proliferating) keratinocytes can be readily observed in sections of wounded skin, the CD45- cells recovered from dissociated tissue samples did not include any BrdU+ cells (preliminary results). Therefore, we suspect that the dissociation protocol we have been using excluded proliferating CD45- cells that are clearly present in the tissue (Fig. A1-5). Indeed, immunofluorescence staining of wounded tissue suggested that most of the Rae1 staining is in the epidermal area (preliminary results), though the specificity of the staining still requires additional confirmation. These data raise the possibility that the keratinocytes, which constitute most of the cells in the epidermal area and incorporate BrdU during wound healing, induce Rae1 expression..

Lymphocytes are another population of cells that actively proliferate *in vivo*, especially after antigenic stimulation. We have not yet documented that proliferating lymphocytes induce high levels of Rae1 or other NKG2D ligands though the analysis

was performed largely by cell surface staining, so it remains possible that Rae1 transcription is highly induced in these cells. This issue should be addressed further in future experiments.

We think it likely that there are circumstances where normal proliferating cells *in vivo* induce Rae1 mRNA, but it appears likely that cell surface protein expression does not generally occur, since it might otherwise target the cells for destruction by immune cells. As mentioned earlier, our hypothesis is that cell surface expression requires signals associated with additional stress pathways or other indications that the cells are undergoing a pathological process. MICA regulation on gut epithelial cells may be one illustration of this hypothesis. MICA is constantly expressed in healthy proliferating gut epithelial cells, but most of the protein is confined intracellular and is not recognizable by NKG2D receptor-expressing immune cells. However, in patients with Celiac disease, MICA is reportedly expressed on the cell surface and renders epithelial cells sensitive to NK killing, leading to some of the pathology associated with this syndrome(Hue, et al., 2004).

Another consideration is that cell surface expression of NKG2D ligands such as Rae1 may in some cases be insufficient to render a cell sensitive to NKG2D-dependent elimination by NK cells. For example, it is reported that adhesion molecules such as ICAM1 are required for NKG2D-dependent lysis (Abdool, et al., 2006). Interestingly, a recent report showed that induction of senescence in a population of tumor cells, resulting from p53 reactivation in vivo, induced significant expression of both ICAM1 and NKG2D ligands, The study showed further that the senescent tumor cells were cleared by immune cells, including NK cells (Xue, et al., 2007). Taken together, this data suggests that induction of Rae1 may by itself be insufficient to render certain cells NK sensitive, and that regulation of adhesion molecules by other mechanisms may also be required in some cases. This could provide another mechanism for immune cells to distinguish malignant cells and normal cells.

Role of the E2F transcription factor family

E2F1 is a unique member of the E2F transcription factor family. Most notably, E2F1 is the strongest inducer of apoptosis among the activating E2F family members. E2F1 fine-tunes proliferation and apoptosis through its impact on the cyclin-Rb pathway, which induces proliferation, and the PI3K pathway, which represses apoptosis. In tumor cells, the balance between these activities is broken due to excessive PI3K activation and frequent mutations in Rb, with the consequence that E2F1 activation favors proliferation over apoptosis. Also, E2F1 can exert a distinct role in the DNA damage response by inducing target genes that function in DNA repair and recombination and DNA damage checkpoints. E2F1 is directly phosphorylated by ATM and acetylation, also driven by the DDR, potentiates the apoptotic functions of E2F1. These studies suggest that E2F1 functions as a sensor that regulates the outcome of different stimuli in cancer. Therefore, E2F1 potentially can be an important link

between the stresses derived from the DDR and the proliferative signals that regulate NKG2D ligand expression.

Although I have shown a clear role of E2F1 in transcription of Rae1, some unpublished data suggested that Rae1 is still expressed in cultured E2F1-/- fibroblasts (Gasser and Raulet, unpublished). A problem with the experiment was that the genetic background of the mice was mixed, and the different alleles of the Rae1 genes may be regulated by different mechanisms (data not shown). It remains unclear whether *Raet1e* is still induced in the absence of E2F1. If so, it does not contradict the claim that E2F plays the key role in regulating *Raet1e*. First of all, many E2F targets are redundantly regulated by E2F1, 2 and 3. Indeed, the proliferation of mouse embryonic fibroblasts (MEF) *in vitro* is completely abrogated only when the cells lack all three E2Fs (Wu, et al., 2001). It would be of interest to determine whether Rae1 expression is abrogated in fibroblasts that lack all three E2Fs.

The existing data bearing on whether E2Fs act redundantly on the Rae1 gene are somewhat perplexing. In preliminary ChIP experiment, E2F2 was bound to the Rae1 promoter in proliferating cells, but E2F3 was not (Fig. 4-6). On the other hand, however, overexpression of E2F2 did not induce Rae1 mRNA or transactivation of the Rae1 promoter, whereas E2F3 overexpression induced modest, but significant Rae1 mRNA (Fig. 4-5, 4-7). A number of experiments are under consideration to resolve these seemingly contradictory data. First, the ChIP data with E2F2 and E2F3 are preliminary and must be repeated. Second, whether the overexpression of E2F3 leads to transactivation of the Rae1 promoter has not yet been tested yet. Considering the finding tht E2F3 induces Rae1 mRNA in transfected cells, it would seem likely that E2F3 does transactivate the Rae1 promoter and does bind the Rae1 promoter. Another possibility is that higher amounts of E2F2 and E2F3 than E2F1 are necessary to activate the Rae1 promoter. In fact, this possibility may be suggested by other data showing that the uninduced levels of E2F2 and E2F3, which are more abundant in normal cells than E2F1 before serum stimulation, are unable to induce E2F target genes (Wu, et al., 2001). Therefore, it should be tested whether higher amounts of E2F2 and E2F3 expression induce Rae1 mRNA and promoter transactivation.

Role of c-Myc in Rae1 transcriptional regulation

A previous study implicated the c-Myc transcription factor in the transcriptional regulation of the Rae1 gene, based on studies of B lymphomas that arise in the Eu-myc transgenic mouse line (Unni, et al., 2008). Those mice express c-Myc in developing B cells, and all the mice develop B or preB lymphomas at several months of age. The study showed that Rae1 protein was abundant at the cell surface of Eu-Myc lymphomas but absent on the B cells of younger "preneoplastic" mice. mRNA analysis showed that Rae1 mRNA is dramatically elevated in Eu-Myc lymphomas, and modestly elevated in the cells of younger preneoplastic mice. A ChIP study showed that c-Myc bound to exon 1 of the Rae1 gene. It was concluded that c-Myc induces *Raet1e* transcription.

It should be noted that the results of a study of B-lymphomas from Eu-Myc mice by our lab deviated in some critical respects from that of Unni et al, in that most of the tumors examined did not express Rae1, and instead, most of the tumors expressed Mult1 (Guerra, et al., 2008). It is not clear why the results of these two studies are discrepant.

If one accepts the results of the Unni study it is still unclear from that data whether c-Myc directly induces transcription of *Raet1e*. The Unni paper shows abundant Rae1 expression in lymphomas from Eu Myc mice, but this does not establish that transgene-encoded Myc directly regulates the *Raet1e* gene. The striking finding in the Unni paper was that Eu-Myc lymphomas expressed high amounts of RAE-1 ε on the cell surface, but it is possible that the role of c-Myc in this system is simply to dramatically accelerate B lymphoma formation, and that the events that induce Rae1 transcription are in fact linked to mutations in other oncogenes, tumor suppressor genes or other genes that are necessary for acquisition of the transformed state. Thus, c-Myc may act indirectly in this system.

On the other hand, the authors also reported that "preneoplastic" B cells in these mice showed elevated Rae1 mRNA, though much less than was observed in B lymphomas. Although this finding might argue for a more direct role of c-Myc in regulating Rae1, it was not ruled out that the elevated Rae1 mRNA was present in a small subset of transformed B cells in the young mice.

The observation that c-Myc associated with the *Raet1e* gene in vivo based on ChIP assays is the most direct evidence that Myc regulates the Rae1 gene. However, no evidence has been reported that c-Myc transactivates the Rae1 promoter or any other regulatory element in the Rae1 gene. Therefore, I tested whether transduction of Myc induces Rae1 expression in non-transformed, primary cells. I observed only a modest induction of Rae1 mRNA by transducing a myc-expressing retrovirus (data not shown). Interestingly, however, I consistently observed that Myc overexpression had a greater impact when the cells were cultured in 10% serum than when cells were cultured in 0% serum. This was true not only for induction of Rae1 but also for induction of another known c-Myc target, cyclin E, suggesting that if Myc does directly induce Rae1 transcription it must cooperate with other factors to do so. In another set of experiments, I determined whether c-Myc transfection transactivated the Rae1 promoter. I found that Myc overexpression could induce less than 2-fold transactivation of the Rae1 promoter. The extent of transactivation did not increase with higher doses of Myc, arguing against the possibility that increased amounts of Myc in tumors could be sufficient to induce Rae1.

Previously, it was shown that Myc interacts with E2F1, and that this interaction is essential for loading E2F1 onto its target sequences in promoters (Leung, et al., 2008). Furthermore, it was shown that Myc is required for the acetylation of histones, by recruiting histone modification enzymes. This observation is consistent with the finding reported by Unni et al, that the acetylation of the Rae1 promoter is increased in Eu-myc transformed cells, assuming that this effect is a direct one. Therefore, it is possible that

the role of Myc in Rae1 induction is to recruit factors such as E2F1 to the Rae1 gene. This hypothesis can be examined by testing whether Myc overexpression transactivates Rae1 promoter constructs in which the E2F1 binding sites are mutated, and by testing whether mutation of the Myc binding site prevents the E2F1-dependent transactivation of the Rae1 promoter. In the context of these proposals, it should be noted that preliminary experiments suggested that Myc and E2F1 co-overexpression did not synergistically induce Rae1 mRNA (data not shown), but it remains possible that only a small amount of c-Myc is necessary to "prime" Rae1 transcription.

It is likely that E2F1's role in regulating Rae1 varies depending on cellular context, including cell type, state of activation, etc. Part of the reason for thinking this is that microarray expression pattern data accumulated with the U74a Mouse Chip (available at URL: http://genome.ucsc.edu/) does not show a strict correlation between Rae1 and E2F1 expression. This may reflect the findings, described earlier, that E2F1 is a versatile protein that can induce different outcomes with different targets in a tissue and context-dependent manner. Another explanation is suggested by a recent report describing a fascinating feature of E2F transcription factors *in vivo*. The data show that although E2F1, 2 and 3 function as activators of proliferation *in vitro* and in developing tissue *in vivo*, they act as repressors when expressed in the absence of other critical signals. Only when Rb is hypophosphorylated or deleted can E2Fs function as activators of targets associated with proliferation (Chong, et al., 2009). Therefore, expression of E2F1 per se does not necessarily indicate that it is present in an activating form, which could account for situations where E2F1 is expressed but Rae1 is not.

Together with previous studies, the findings reported here indicate that there are multiple pathways leading to the induction of NKG2D ligands. In some cells or circumstances, these levels of regulation may serve as serial checkpoints, regulated by distinct stimuli, to ensure that ligands are induced only in diseased cells and not in normal cells. Understanding the cooperation of the individual signals and stress pathways and their contribution in different tissues or tumors will provide important insights concerning the biology of NKG2D ligand regulation. The data presented in this thesis demonstrate that the E2F transcription factors, critical regulators of cell proliferation, induce transcription of the gene encoding the *Raet1e* NKG2D ligand, providing an important link between the proliferative status of cells and the immune response. Further analysis of E2Fs role may provide new approaches for understanding how oncogenic stress leads to tumor elimination by immune cells.

Appendix

Appendix1. NKG2D in the context of wound healing

Previously, one of the NKG2D ligands, H60c, was shown to be exclusively expressed in the skin. Skin wounding induced H60c transcript levels substantially (Whang, et al., 2009). Furthermore, Rae1 transcripts were elevated in wounded skin (see below). Interestingly, the most abundant immune cells in the epidermal layer of the skin, dendritic epidermal T cells (DETC), constitutively express NKG2D. Studies suggest that DETCs play an important role in promoting would healing (Jameson, et al., 2002). These findings raise the possibility that NKG2D may play a role in wound healing, potentially as a result of engagement of NKG2D with H60c or Rae1.

Role of NKG2D in wound healing

To test whether NKG2D plays a role in wound healing, the rate of wound healing was measured in WT B6 and NKG2D deficient mice. One pair of mice were wounded with a punch that generates 2mm diameter holes on the back every day for 4 days, and on the fifth day all the mice sacrificed and skin is pilled off from the back. Skins that were laid flat on the plate were photographed and the size of wound were analyzed by ImageJ program. Each mouse received two wounds, and the wound sizes were averaged. In three independent experiments, we consistently observed delayed wound closure in NKG2D deficient mice (Fig. A1-1 *A*). Although the differences seem to be small, it is statically significant and comparable to the published data from Jameson et al,. 2002 (Fig.A1-1 *B*), which showed wound closure in gdTCR-/- mice is delayed compared to WT mice.. These data indicate that NKG2D plays a role in wound healing, presumably indicating that NKG2D-ligand interactions promote DETC activation and thereby promote wound healing.

Recruitment of CD45 positive cells to wound sites

During the wound healing process, non-lymphoid cells including keratinocytes and fibroblasts proliferate in response to serum growth factors (Singer and Clark, 1999). Furthermore, a large number of CD45+ immune cells are recruited to the wound sites (Singer and Clark, 1999). Flow cytometry experiments I performed confirmed that whereas only 20-30% of dissociated skin cells were CD45+ in unwounded samples, nearly 90% of the cells were CD45+ in wounded samples (Fig. A1-2). The percentage of CD45+ cells in the wounded samples was similar whether the affected mice were NKG2D WT or NKG2D KO, suggesting there was not a major defect in immune cell recruitment as a result of NKG2D deficiency. However, preliminary data suggests that the recruitment of some subsets of immune cells, for example NK cells and T cells other than DETC (CD3+ Vg3- cells), were delayed in NKG2D KO mice (data not shown). Additional studies will be necessary to clarify the influence of NKG2D on the cell types that are recruited to wounded skin in detail.

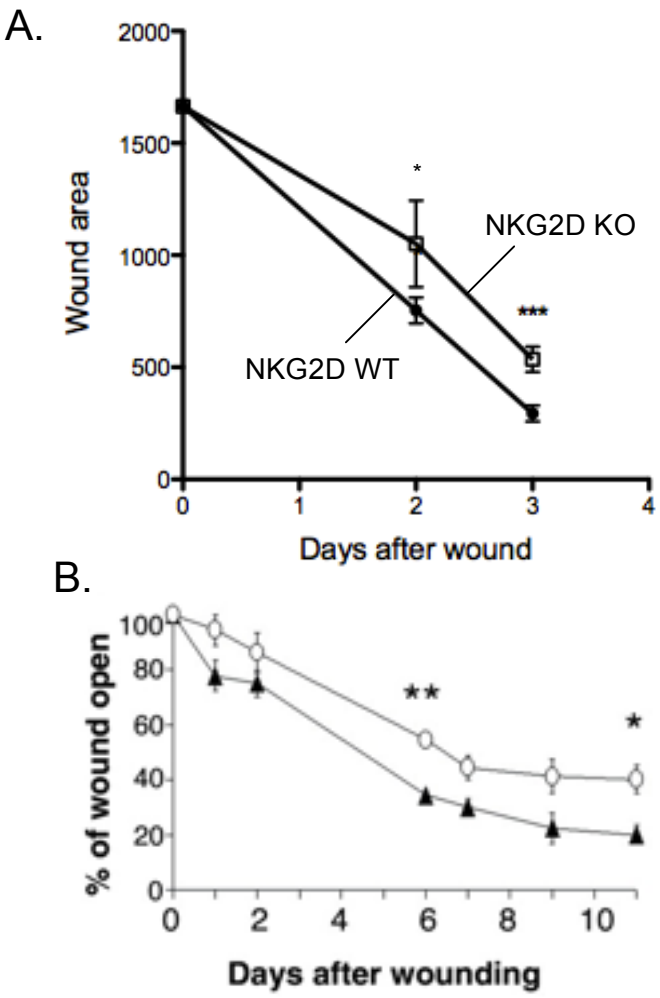

Fig.A1-1. Wound healing is delayed in NKG2D-deficient mice. A. NKG2D-deficient (KO) mice and WT littermates were wounded with a 2mm punch on the back to generate 2 wounds per mouse. On the indicated days, mice were sacrificed and the wounded skin was laid flat on a surface and photographed. The sizes of wounds were determined using ImageJ. *=$p<0.005$. ***=$p<0.0005$. Data in this figure are representative of three independent experiments. **B.** Figure from published paper (Jameson, et al., 2002) is shown as a comparison. Open circles represent wounds from gd TCR-/- mice and closed triangles represent wounds from WT control mice.

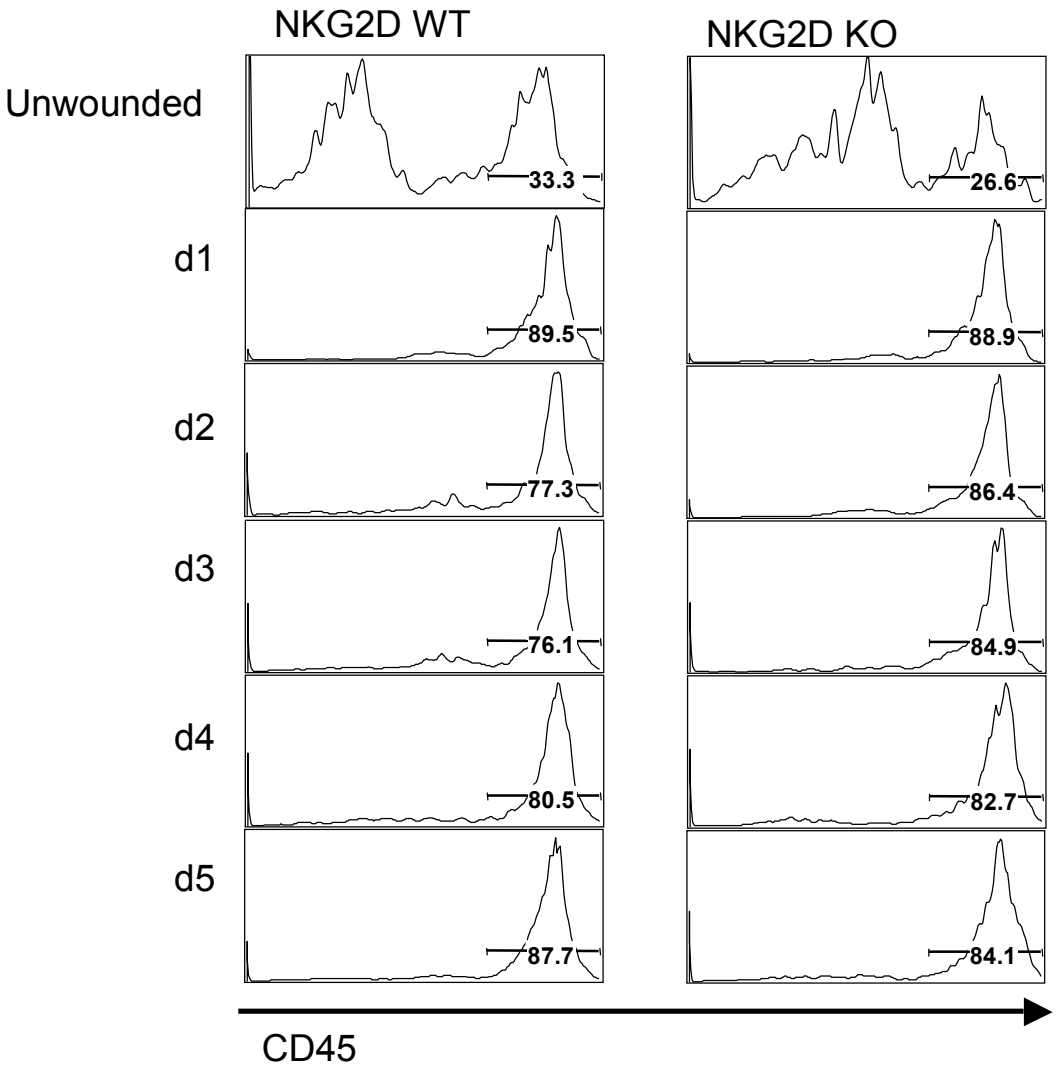

Fig.A1-2. Immune cell recruitment to wound sites occurs in NKG2D deficient mice. Wounds from NKG2D-deficient mice (KO) and WT littermates were enzymatically dissociated and stained for CD45 expression for flow cytometry analysis. Data in this figure are representative of at least two independent experiments.

Increased amounts of Raet1e RNA in skin wounds

The wound-healing process activates a similar transcription program as when fibroblasts are exposed to serum-induced proliferation in fibroblasts, as cells become exposed to serum that is usually sequestered from. My results in Chapter 3 showed that fibroblasts exposed to serum induce expression of RAE-1 ε. These considerations led me to ask whether wound healing induces another NKG2D ligand *Raet1e* at the sites of wounds *in vivo*.

To test whether Rae1 is also induced in wounded skin tissue, RNA was harvested from skin tissues 1~3 days after wounding. Tissue from wounded and unwounded control mouse was ground in the presence of liquid nitrogen, followed by RNA extraction with Trizol. As shown in Fig. A1-2, *Raet1e* RNA was significantly induced in wounded skin tissue from both NKG2D WT and KO wounded skin tissue mice one day after wounding and the elevated levels were sustained for at least 3 days from the first day of wound.

To test whether RAE-1 ε protein is expressed on the cell surface in wounded tissue, wound tissues were frozen in OCT blocks and cut into 7~10 μM sections, and stained with fluorescent anti-RAE-1 ε antibody for immunofluorescence. We observed Rae1 staining with antibody in most ares of the epidermis and to some extent in the dermis (data not shown). However, wounds from BALB/C mice, where RAE-1 ε expression has not been detected in any experimental conditions, also stained with RAE-1 ε antibody although the signal was less intense, which raised the possibility that the staining could be non-specific.

As another more sensitive approach, which can also determine whether Rae1 is expressed on the cell surface, single cell suspensions of dissociated skin from wounded tissues were stained with RAE-1 ε antibody for flow cytometry analysis. Preliminary data suggest that there was no specific RAE-1 ε staining of wounded B6 tissues compared to wounded BALB/c tissues (data not shown). However, whether Rae1 protein is expressed intracellular is remain unclear.

To summarize these results, although *Raet1e* RNA was induced upon wounding, we have so far failed to observe increased amounts of RAE-1 ε protein cell surface expression in wounded tissue. As a caveat, it is possible that cells in wounded tissues are rendered stickier that cells in unwounded tissue, resulting in higher background staining. The higher backgrounds may mask specific staining.

The results in Fig. A1-1 indicate a role for NKG2D in wound healing, suggesting that NKG2D ligands are present in wounded tissue. As H60c RNA has also been shown to be induced in wounded skin samples, it will be valuable to test in future experiments whether H60c is expressed on the surface of cells in wounded tissues.

Cell types containing Raet1e mRNA in wounded tissues

To investigate the cell types in which *Raet1e* RNA is found in wounded samples, skin samples were enzymatically dissociated into single cell suspensions, and the populations were separated by cell sorting into CD45+ (lymphohematopoietic) and CD45- fractions. NKG2D transcripts (Fig. A1-4. *A*) and CD104 (a keratinocyte marker) transcripts (Fig. A1-4. *B*) were largely confined to the CD45+ and CD45- populations, respectively, demonstrating the purity of the two populations.

Surprisingly, most *Raet1e* transcripts were present in the CD45+ population. The CD45- population contained lower amounts or *Raet1e* transcripts, that were not significantly elevated compared to the levels in unseparated unwounded skin samples (Fig. A1-4. *C*). The identify of the CD45+ cell types that express Rae1 in wounded skin remains to be determined in future experiments. One possibility is that the Rae1 mRNA is present in macrophages. Macrophages have been shown to induce Rae1 RNA upon stimulation with toll-like receptor ligands (Hamerman, et al., 2004), and it is reported that self-nucleic acid is released in wound sites, and can cause activation of TLR7 and 9 (Gregorio, et al.). Therefore, it will be important to test whether F4/80+ cells (macrophages) in cell samples from skin wounds contain elevated Rae1 mRNA.

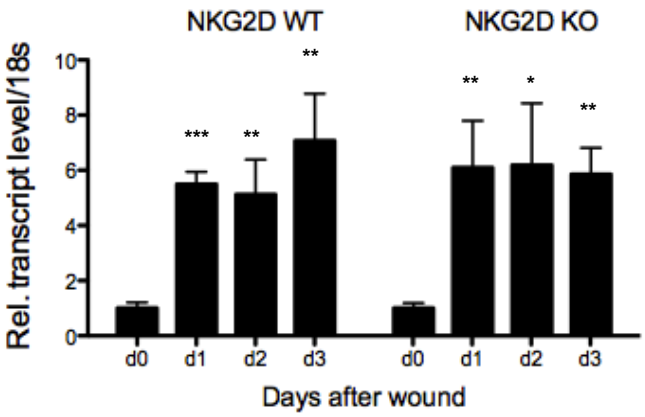

Fig.A1-3. RAE-1 ε is increased in wounded skin. Wounds from NKG2D WT or KO skin tissue were ground in the presence of liquid nitrogen, and RNA was harvested by Trizol. Relative transcript amounts were determined by quantitative PCR and normalized to 18s RNA amounts. Data in this figure are representative of three or more independent experiments.

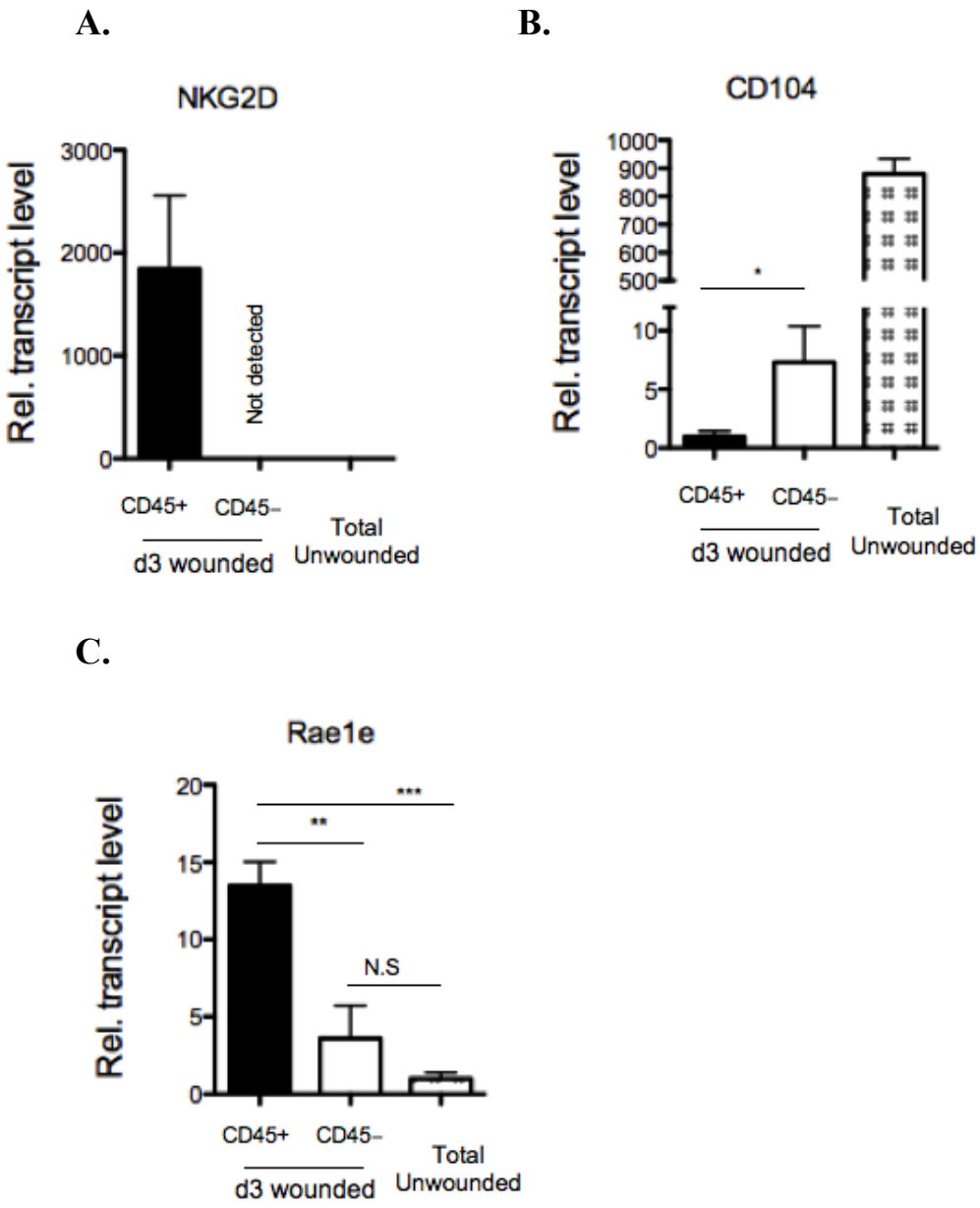

Fig.A1-4. RAE-1 ε mRNA is predominantly contained in CD45+ cells in wounded tissue samples. Wounded skin samples (3 days post wounding) were dissociated, sorted into CD45+ and CD45- fractions, and RNA was extracted. cDNA preparations were subjected to quantitative PCRs for RAE-1 ε, NKG2D or CD104. and the results were normalized to 18s rRNA amounts. As a control, total unwounded skin tissue was used to make RNA. Data in this figure are representative of two independent experiments.

Visualization of proliferating cells in wounded skin samples

In Chapter 3 it was shown that Rae1 mRNA and protein are induced in proliferating cells. To examine whether the induction of Rae1 mRNA in wounded skin was correlated with cell proliferation, BrdU was injected into wounded mice one day before sacrifice. A segment of wounded tissue was prepared that included a small rim of tissue around the wound. Immunofluorescence staining of BrdU on frozen sections was performed. It was not possible to costain with Rae1 and H60 antibodies, because the harsh permeabilization and fixation conditions used for BrdU staining can destroy the epitopes recognized by Rae1 and H60 antibodies. The CD45 epitope, in contrast, was not destroyed by this staining procedure. Co-staining of CD45 and BrdU in frozen sections showed that most of the cells that proliferated between day 2 and day 3 following wounding were CD45-negative cells (Fig. A1-5). Because most cells with Rae1 mRNA are CD45+ (Fig.A1-4), the data suggest that RAE-1 ε induction in healing wounds is not correlated with cell proliferation. Apparently, induction of Rae1 mRNA in CD45+ cells in wounded tissue occurs by a mechanism independent of cell proliferation. The analysis of BrdU vs CD45 expression should be confirmed by flow cytometry, a more sensitive method, and should include conditions where mice are labeled with BrdU between day 0 and day 2.

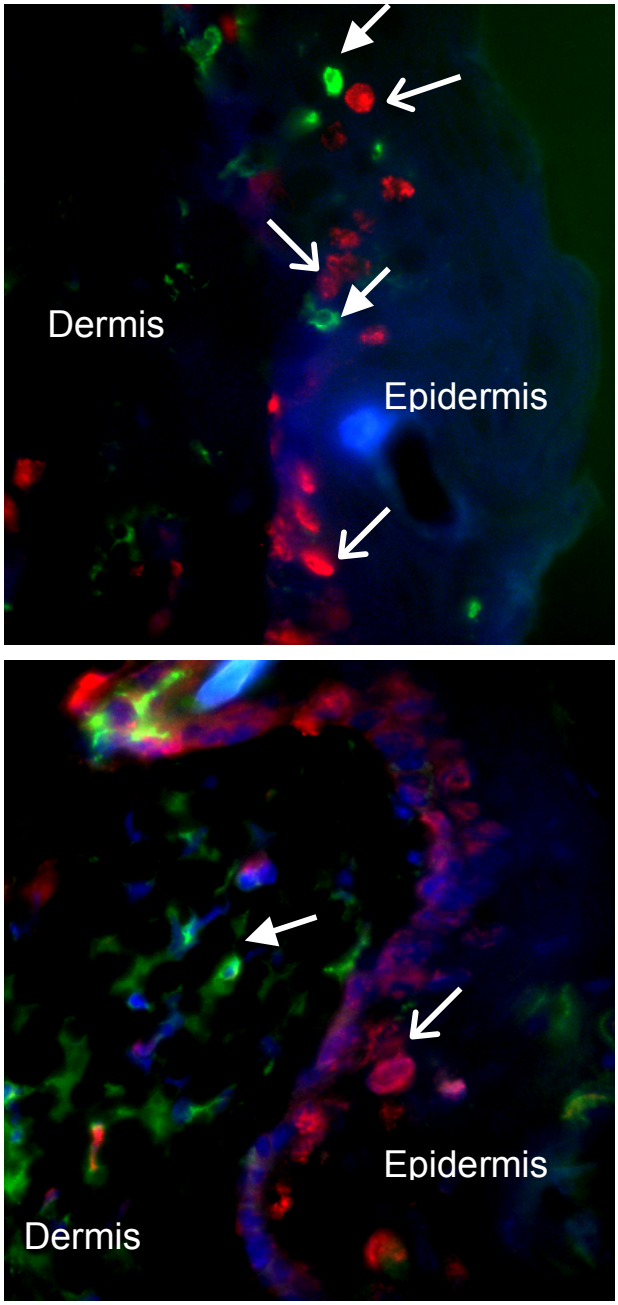

Fig. A1-5. Non-lymphoid cells proliferate during wound healing. BrdU was injected on day 2 after wounding, and mice were sacrificed one day later. Wounded tissues were frozen in O.C.T and cut into 7~10uM sections, which were stained with CD45 (Green) and BrdU (Red) antibodies, and DAPI (Blue). Due to the harsh permabilization conditions for BrdU staining, the DAPI staining is not well defined. The two images are representative of more than 10 sections examined. Examples of clear CD45 staining are shown with triangular arrowheads, and examples of clear BrdU staining are shown with barbed arrowheads.

Summary

Wound healing is a complex process involving various cell types, including immune cells recruited by chemokines and cytokines. This chapter addressed a possible role of NKG2D and its ligands in wound healing. An important finding was that wound closure was delayed in NKG2D-deficient mice. Although there was no major defect in the quantity of immune cells recruited in the absence of NKG2D, preliminary data suggest that the recruitment of specific subsets, specifically NK cells and CD3+ cells, may be delayed. It remains unclear which NKG2D+ cells are involved in wound closure, though DETC are likely candidates based on previous evidence that mice lacking these cells (TCRd-/- mice) are characterized by delayed wound healing.

The expression of NKG2D ligands in wounded skin was investigated. New evidence showed that RAE-1 ε mRNA was increased upon wounding, adding to published evidence that H60c mRNA is increased in wounded skin. The Rae1 mRNA was confined to the immune cell population. Additional data suggested that immune cells in the wound had undergone minimal proliferation, however, arguing that Rae1 induction may be independent of proliferative signals in the context of wound healing.

Appendix2. Role of NKG2D on CD8 T cells in tumor immunity

In human CD 8 T cells express NKG2D, but in mice, it is induced by activation. Several studies have shown a role of NKG2D as a costimulatory molecule for CD 8 T cell responses. NKG2D engagement enhanced in vitro responses, especially when CD3 signaling is limiting (Jamieson Immunity 2002). Furthermore, NKG2D could stimulate CD8 effector functions even with no engagement of the T cell receptor in certain pathological process (Meresse, et al., 2004). On the contrary, Lanier's group has reported that NKG2D ligands on tumor cell line did not result in T cell mediated rejection nor memory formation, and NKG2D did not costimulate CD8 T cells *in vitro* (Cerwenka, et al., 2001), (Ehrlich, et al., 2005)). Therefore, there is still a controversy in the role of NKG2D on CD 8 T cells.

With NKG2D deficient mice available, it provides a more defined approach to address the question. To determine whether NKG2D enhance CD 8 T cell function, specifically in tumor immunity, we decided to monitor tumor rejection by T cells from NKG2D WT or KO background *in vivo*. For specific T cell response, we utilized OT-1 TCR transgenic mouse and RMA tumors that express its cognate antigen, OVA peptide (SIINFEKL) and/or NKG2D ligand, Rae1 beta by transducing MSCV-vector that express each genes. To ensure that T cells, but not other immune cells lack NKG2D, we decided to transfer OT-1 NKG2D WT or KO splenocytes into B6 mice and inject tumor and observe the rejection.

Firstly, the dose of RMA-Rae1b tumors was titrated in B6 (Fig. A2-1). The dose of tumors that were not rejected by host immune cells, but did not grow too fast before T cell immunity can intervene, was chosen, which was 10^6 cells. However, RMA-Rae1b that also transduced with OVA was rejected at this dose (Fig.A2-2), which suggests that expression of OVA renders this RMA tumor cells recognized by host immune cells. The rejection seems to be T or B cell mediated considering that it took more than 8 days before it was rejected.

Therefore, I increased the amount of RMA-Rae1b OVA tumor cell numbers to 3×10^6 cells to avoid early rejection. I also test whether transfer of Ly45.1 OT-1 TCR transgenic, NKG2D WT CD8 T cells to tumor injection could facilitate tumor rejection in this set of experiment. 5×10^4 or 5×10^5 CD8 T cells that were sorted by magnetic beads from OT-1 TCR transgenic splenocytes were intravenously injected 2 days prior to tumor injection. As shown in Fig. A2 - 3, tumor cells grew bigger at this dose, but they were eventually rejected at day 9. However, with T cell transfer, the tumor grew much smaller and rejected earlier compared to no transfer control , even with lower dose of T cells. Nevertheless, tumors were successfully rejected even without OT-1 T cell transfer by host immune cells, which suggests that NKG2D WT host immune cells, including CD8 T cells, are actively participating in the rejection of tumors. Tumor in one of the mice that received T cell transfer relapsed at d15. Examination of lymphocytes present in the blood by staining Ly45.1 and Ly45.2 showed that mouse that had relapsed tumor completely lost OT-1 T cells, whereas other mice had OT-1 T cells (data not shown). This suggested that OT-1 T cells were actively suppressing tumorigenesis, however, more mice should be tested to confirm this result.

In the next experiment, I increased the number of RMA-Rae1b OVA tumor cells to 10^7 cells. At this dose, the tumor was not rejected by host immune cells. In the contrary, mice that received OT-1 T cell transfer efficiently rejected tumor by d15. However, this protection was not dependent on NKG2D, as NKG2D KO CD8 T cell transfer was sufficient to reject the tumor cells (Fig. A2-4). This preliminary experiment suggests that NKG2D does not play a role in tumor rejection by antigen specific CD8 T cells. However, this should be repeated to confirm this result. Additionally, although tumor was rejected by transferred T cells, it is still possible that NKG2D sufficient host CD8 T cells could play a role in this process. Therefore, this experiment should be repeated in Rag-deficient or CD8 T cell-deficient background to ensure that the CD8 T cell response against tumor can be restricted to the transferred cells.

Most of tumor antigen- TCR affinity is usually low, as most of them are self-protein. However, OVA peptide- OT-1 affinity is unusually high, which fail to recapitulate physiological condition of tumor antigen-receptor interaction. As OVA peptide variants, which has lower affinity to OT-1 TCR has been well characterized, tumors that express lower affinity antigen can be tested by transducing variant OVA peptide expressing virus, and investigate the role of NKG2D in rejection of tumors. It will be of interest to find out the role of NKG2D in this condition, which better reflects the physiological condition.

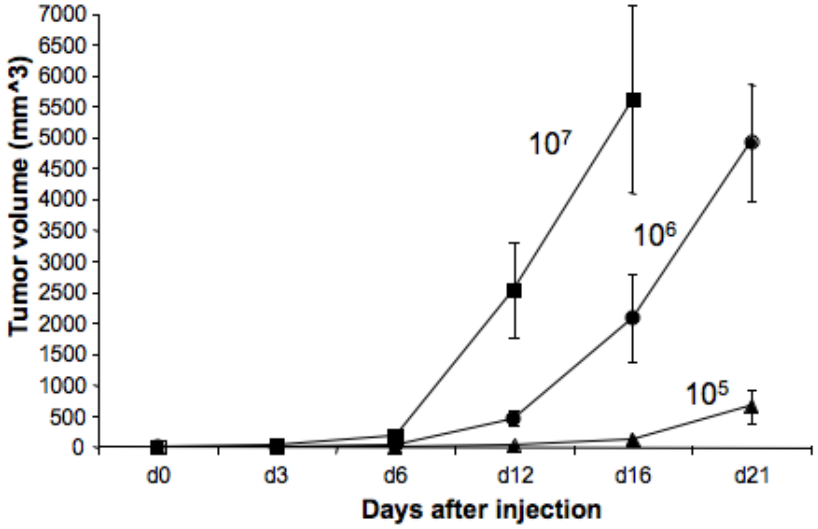

Fig. A2-1. RMA-Rae1b tumor cell growth in B6 mice. Indicated number of RMA-Rae1b was subcutaneously injected into the left flank of B6 mice and the tumor size was monitered every 3~4 days. n=3~4.

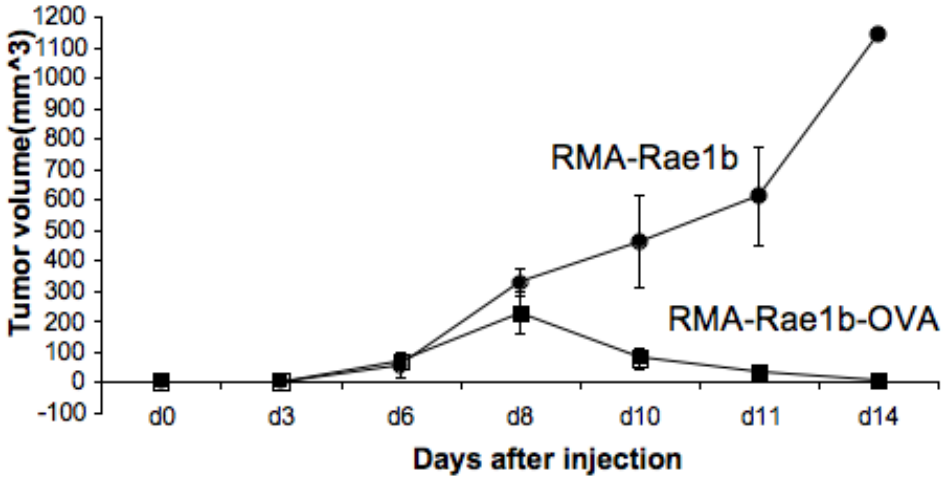

Fig. A2-2. RMA-Rae1b-OVA, but not RMA-Rae1b tumor cells were rejected.
10^6 tumor cells were subcutaneously injected into the left flank of B6 mice and the tumor size was monitered every 2~3 days. n=4.

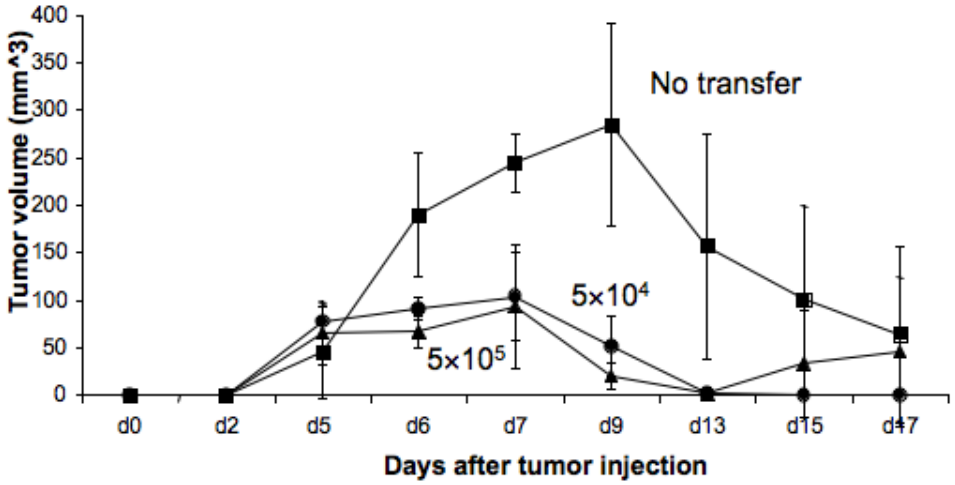

Fig. A2-3. Transferred OT-1 CD8 T cells accerate rejection of RMA-Rae1b-OVA tumor cell lines. $3X10^6$ tumor cells were subcutaneously injected into the left flank of B6 mice and the tumor size was monitered every 2~3 days. Some groups received $5X10^4$ (circle) or $5X10^5$ (triangle) OT-1 CD8 T cells intravenously 2 days prior to tumor injection. n=4.

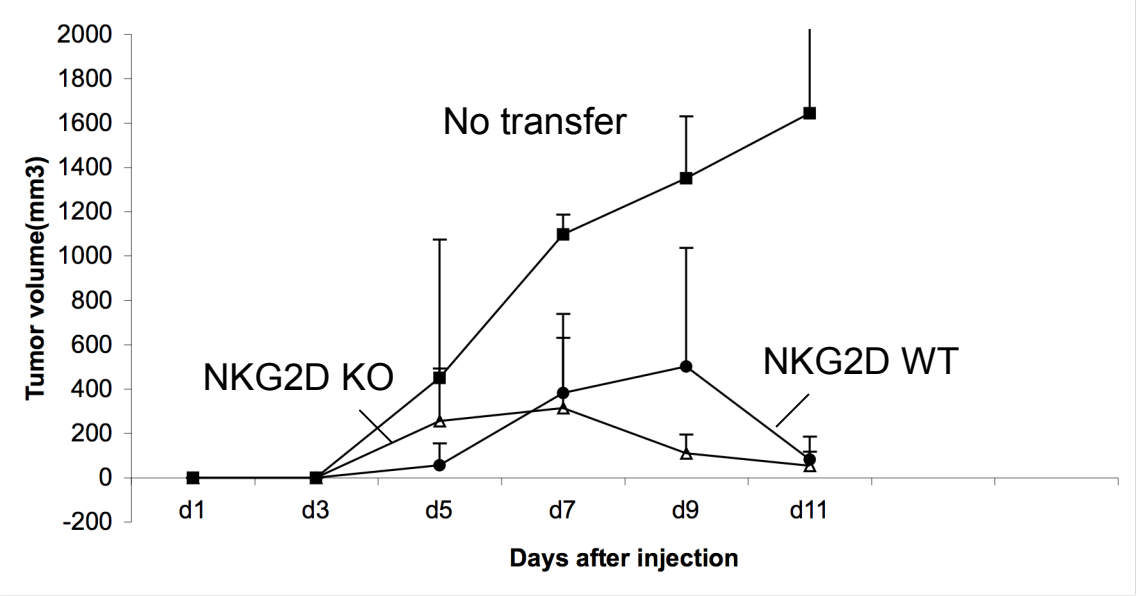

Fig. A2-4. Transferred OT-1 CD8 T cells mediate rejection of RMA-Rae1b-OVA tumor cells independent of NKG2D. 10^7 tumor cells were subcutaneously injected into the left flank of B6 mice and the tumor size was monitered every 2~3 days. Some groups received indicated number of NKG2D WT (filled circle) or NKG2D KO (open triangle) OT-1 CD8 T cells intravenously 2 days prior to tumor injection. n=3~4.

LIST OF ABBREVIATION

ChIP Chromatin immunoprecipitation
CLP Common lymphoid progenitors
DCFDA 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
DMEM Dulbecco's Modified Eagle Medium
EGF Epidermal growth factor
FCS Fetal calf serum
HDL High density lipoprotein
LAK Lymphokine-activated killer cells
MFI Mean fluorescence intensity
NAC N-acetyl-l-cysteine
ORF Open reading frame
PVP Polyvinylpyrrolidone
ROS Reactive oxygen species
TESS Transcription Element Search Software

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