What can you do with a luciferase Reporter Assay?

Signaling Pathway Analysis Applications

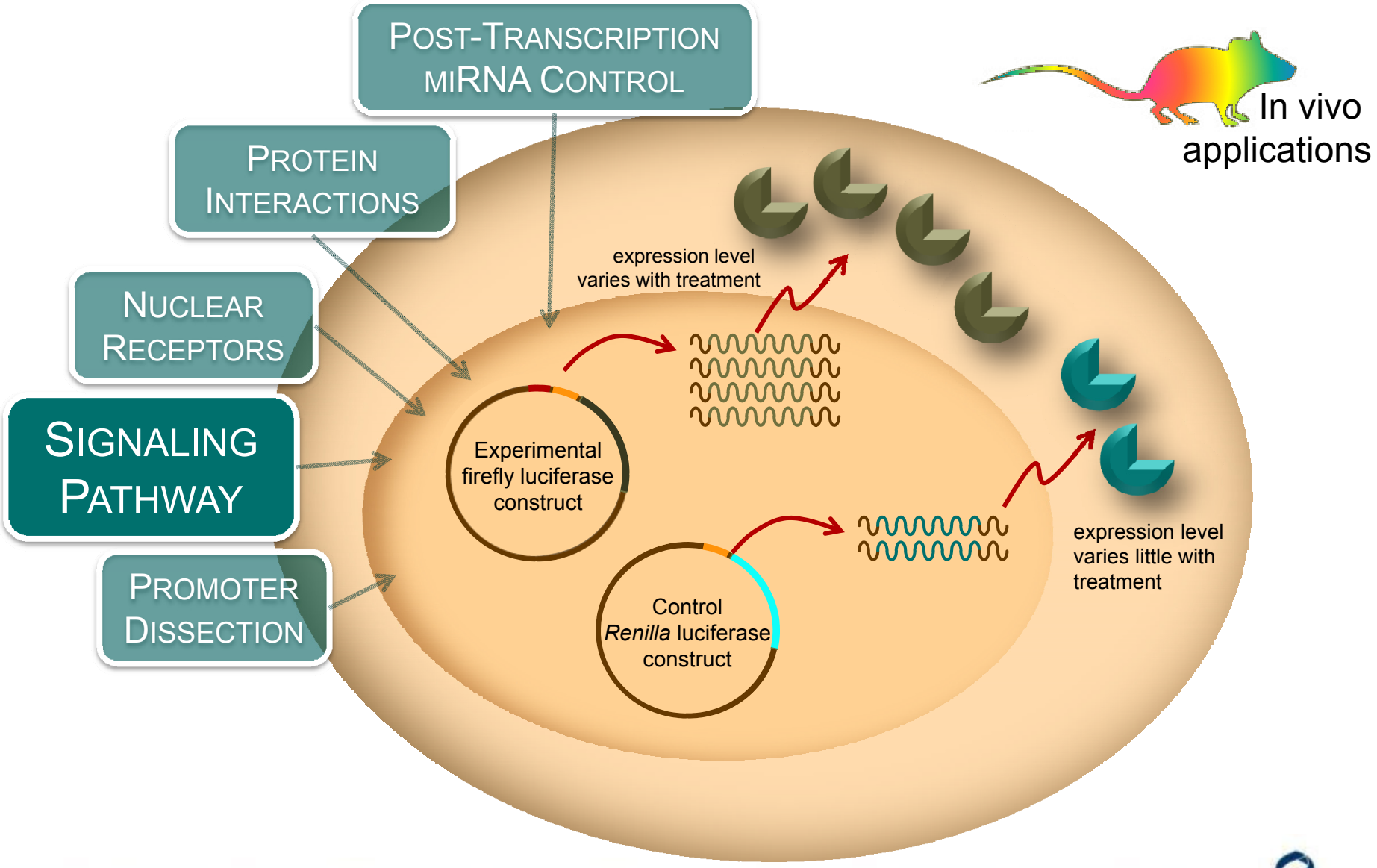
Presented Fall 2009

Click the icon in the upper left hand corner to view speaker notes for slides.

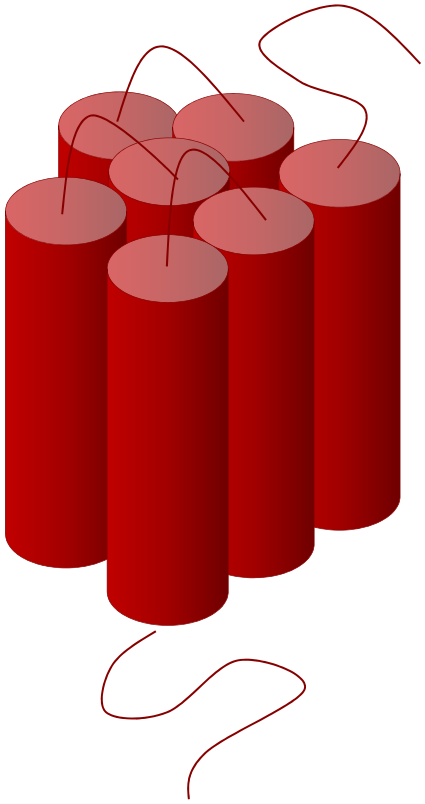
Have a question? Ask a Scientist

Promega

Application Overview



What signaling pathway does my protein use?



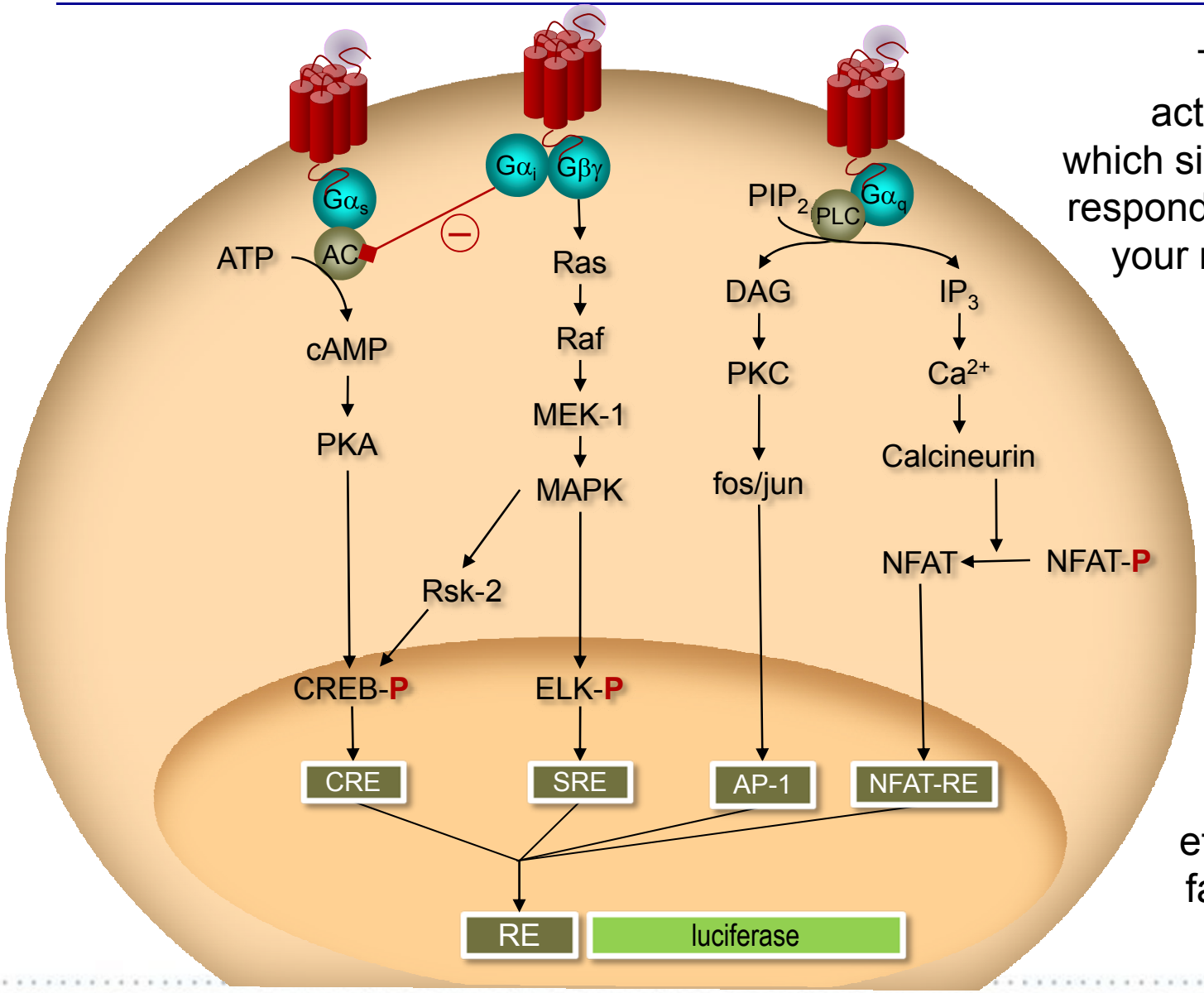
You've identified a novel GPCR receptor.

- Which pathway does it use for signal transduction?
- Which G protein does it utilize?

Synthetic promoter constructs can help you identify which pathways are involved

Promega

Investigate signal transduction pathways

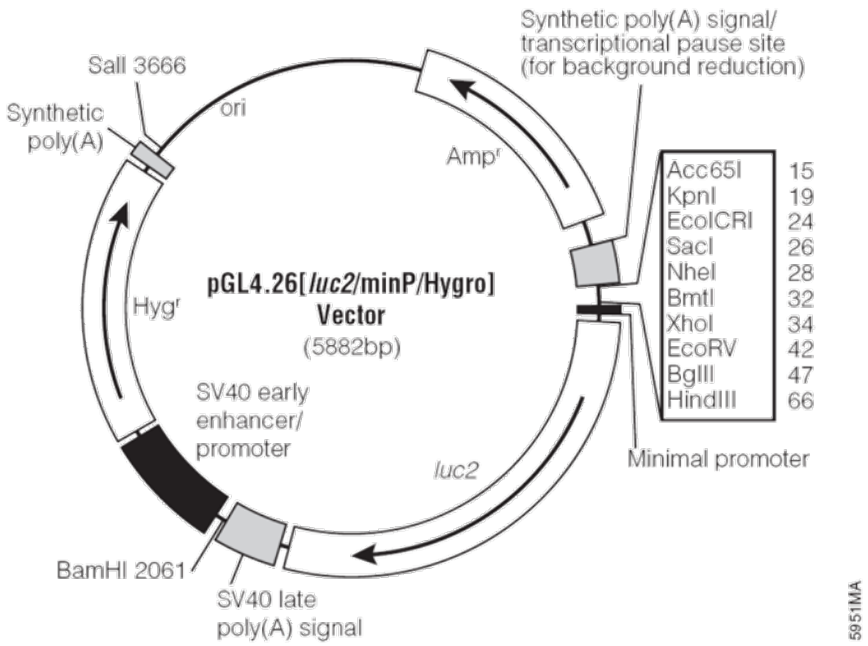


The reporter assay activation can tell you which signaling pathway is responding to activation of your receptor of interest

Now you can use inhibitors, siRNA, etc., to define which factors are involved

Promega

Easily create response element reporters

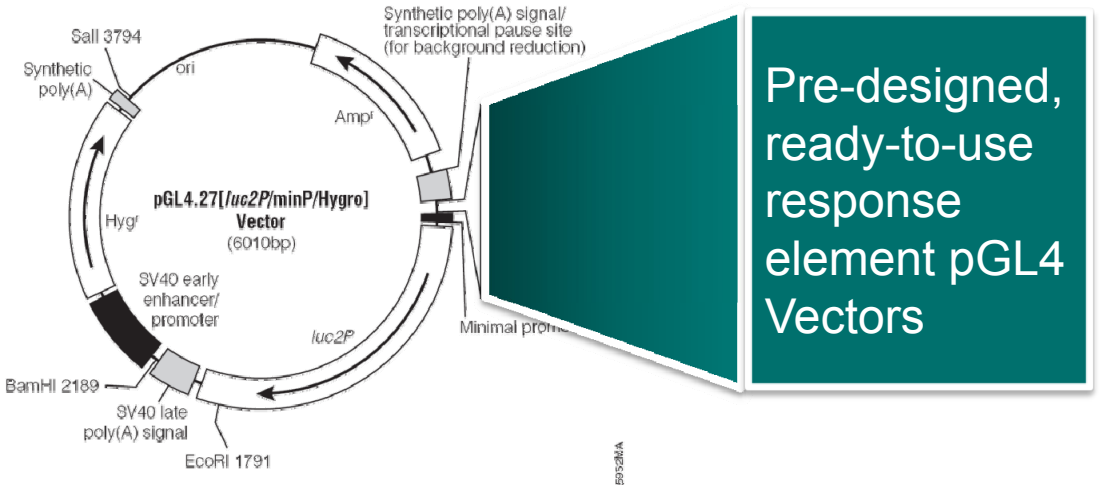


- Response elements for many transcription factors are known
- Response elements can be placed upstream of a minimal promoter to create synthetic reporter constructs for monitoring signal transduction

RapidResponse™ versions available

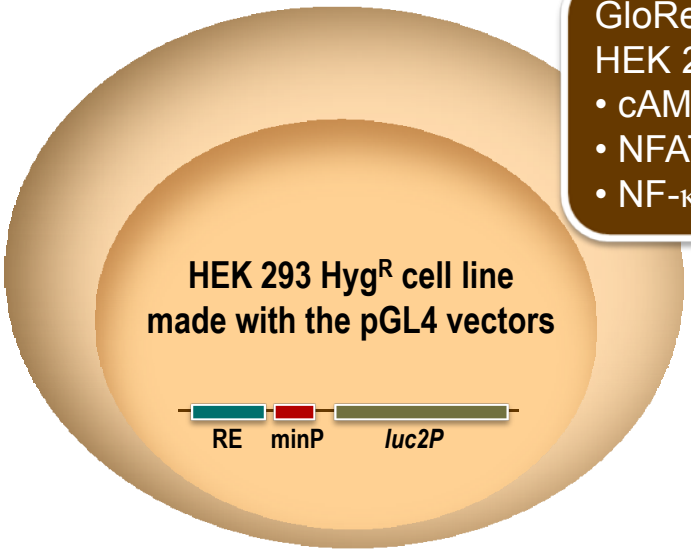
Promega

Tools to speed your research

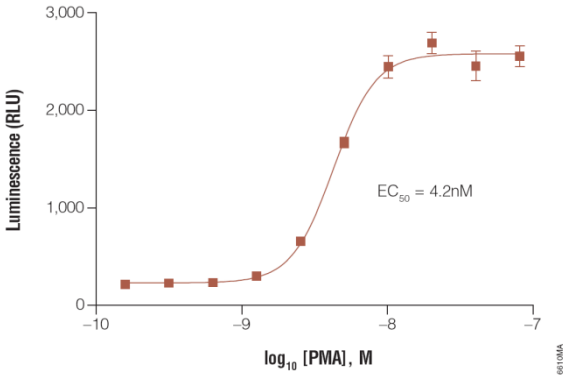


Pre-designed, ready-to-use response element pGL4 Vectors

Response Element	Signaling Pathway	pGL4 Vector
cAMP	cAMP/PKA	pGL4.29
NFAT Nuclear Factor of Activated T-cells	Ca^{2+}/Calcineurin	pGL4.30
NF-κB	NF-κB	pGL4.32
Serum Response	MAP/ERK	pGL4.33
Serum Response Factor	RhoA	pGL4.34



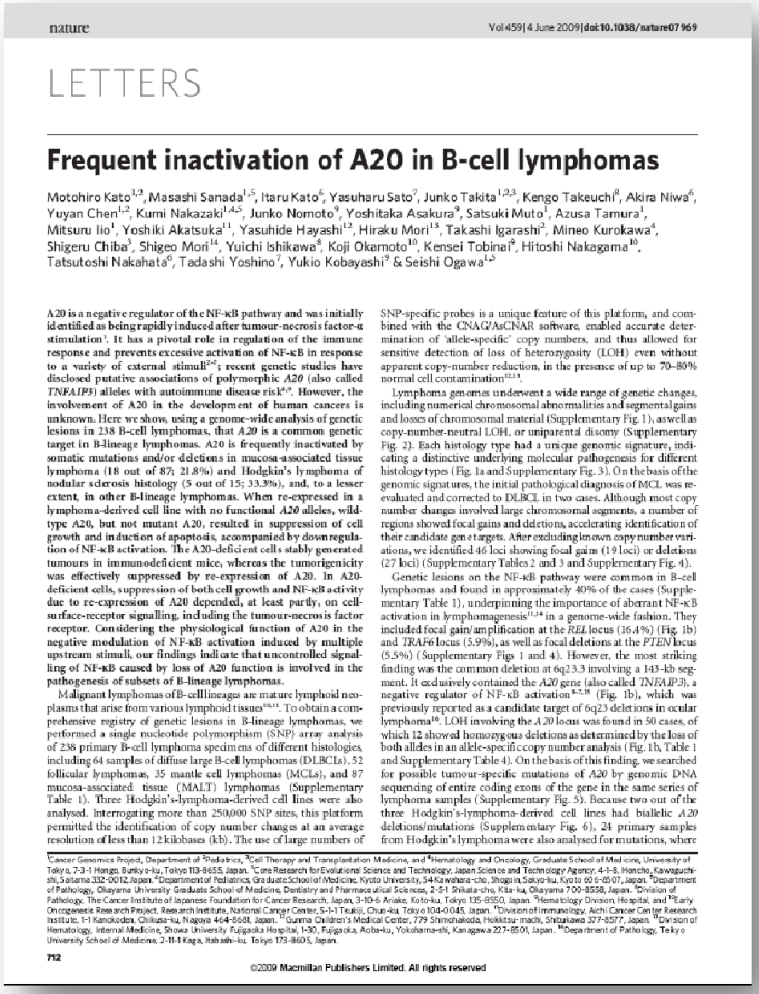
GloResponse™ HEK 293 cell lines:
- cAMP RE
- NFAT RE
- NF-κB RE



Dose response curve
GloResponse NFAT-RE-*luc2P*
HEK293 Cell Line

Promega

Case Study: A20 addition controls lymphoma



nature Vol 459 | 4 June 2009 | doi:10.1038/nature07969

LETTERS

Frequent inactivation of A20 in B-cell lymphomas

Motohiro Kato[1,2], Masashi Sanada[1,3], Itaru Kato[4], Yasuharu Sato[2], Junko Takita[1,2,3], Kengo Takeuchi[5], Akira Niwa[6], Yuyan Chen[1,2], Kumi Nakazaki[1,4,5], Junko Nomoto[3], Yoshitaka Asakura[3], Satsuki Muto[1], Azusa Tamura[1], Mitsuru Iio[1], Yoshiki Akatsuka[1,3], Yasuhide Hayashi[1,2], Hiraku Mori[1,3], Takashi Igarashi[2], Mineo Kurokawa[4], Shigeru Chiba[5], Shigeo Mori[1,4], Yuichi Ishikawa[8], Koji Okamoto[10], Kensei Tobinai[9], Hitoshi Nakagama[10], Tatsutoshi Nakahata[6], Tadashi Yoshino[7], Yukio Kobayashi[3] & Seishi Ogawa[1,2]

A20 is a negative regulator of the NF-κB pathway and was initially identified as being rapidly induced after tumour-necrosis factor-α stimulation[1]. It has a pivotal role in regulation of the immune response and prevents excessive activation of NF-κB in response to a variety of external stimuli[2–4]; recent genetic studies have disclosed putative associations of polymorphic *A20* (also called *TNFAIP3*) alleles with autoimmune disease risk[5,6]. However, the involvement of A20 in the development of human cancers is unknown. Here we show, using a genome-wide analysis of genetic lesions in 238 B-cell lymphomas, that *A20* is a common genetic target in B-lineage lymphomas. A20 is frequently inactivated by somatic mutations and/or deletions in mucosa-associated tissue lymphoma (18 out of 87; 21.8%) and Hodgkin's lymphoma of nodular sclerosis histology (5 out of 15; 33.3%), and, to a lesser extent, in other B-lineage lymphomas. When re-expressed in a lymphoma-derived cell line with no functional *A20* alleles, wild-type A20, but not mutant A20, resulted in suppression of cell growth and induction of apoptosis, accompanied by down regulation of NF-κB activation. The A20-deficient cells stably generated tumours in immunodeficient mice, whereas the tumorigenicity was effectively suppressed by re-expression of A20. In A20-deficient cells, suppression of both cell growth and NF-κB activity due to re-expression of A20 depended, at least partly, on cell-surface-receptor signalling, including the tumour-necrosis factor receptor. Considering the physiological function of A20 in the negative modulation of NF-κB activation induced by multiple upstream stimuli, our findings indicate that uncontrolled signalling of NF-κB caused by loss of A20 function is involved in the pathogenesis of subsets of B-lineage lymphomas.

Malignant lymphomas of B-cell lineages are mature lymphoid neoplasms that arise from various lymphoid tissues[6,7]. To obtain a comprehensive registry of genetic lesions in B-lineage lymphomas, we performed a single nucleotide polymorphism (SNP) array analysis of 238 primary B-cell lymphoma specimens of different histologies, including 64 samples of diffuse large B-cell lymphomas (DLBCLs), 52 follicular lymphomas, 35 mantle cell lymphomas (MCLs), and 87 mucosa-associated tissue (MALT) lymphomas (Supplementary Table 1). Three Hodgkin's-lymphoma-derived cell lines were also analysed. Interrogating more than 250,000 SNP sites, this platform permitted the identification of copy number changes at an average resolution of less than 12 kilobases (kb). The use of large numbers of SNP-specific probes is a unique feature of this platform, and combined with the CNAG/AsCNAR software, enabled accurate determination of 'allele-specific' copy numbers, and thus allowed for sensitive detection of loss of heterozygosity (LOH) even without apparent copy-number reduction, in the presence of up to 70–80% normal cell contamination[8,9].

Lymphoma genomes underwent a wide range of genetic changes, including numerical chromosomal abnormalities and segmental gains and losses of chromosomal material (Supplementary Fig. 1), as well as copy-number-neutral LOH, or uniparental disomy (Supplementary Fig. 2). Each histology type had a unique genomic signature, indicating a distinctive underlying molecular pathogenesis for different histology types (Fig. 1a and Supplementary Fig. 3). On the basis of the genomic signatures, the initial pathological diagnosis of MCL was re-evaluated and corrected to DLBCL in two cases. Although most copy number changes involved large chromosomal segments, a number of regions showed focal gains and deletions, accelerating identification of their candidate gene targets. After excluding known copy number variations, we identified 46 loci showing focal gains (19 loci) or deletions (27 loci) (Supplementary Tables 2 and 3 and Supplementary Fig. 4).

Genetic lesions on the NF-κB pathway were common in B-cell lymphomas and found in approximately 40% of the cases (Supplementary Table 1), underpinning the importance of aberrant NF-κB activation in lymphomagenesis[10–14] in a genome-wide fashion. They included focal gain/amplification at the *REL* locus (16.4%) (Fig. 1b) and *TRAF6* locus (5.9%), as well as focal deletions at the *PTEN* locus (5.5%) (Supplementary Figs 1 and 4). However, the most striking finding was the common deletion at 6q23.3 involving a 143-kb segment. It exclusively contained the *A20* gene (also called *TNFAIP3*), a negative regulator of NF-κB activation[4,5,8] (Fig. 1b), which was previously reported as a candidate target of 6q23 deletions in ocular lymphoma[16]. LOH involving the *A20* locus was found in 50 cases, of which 12 showed homozygous deletions as determined by the loss of both alleles in an allele-specific copy number analysis (Fig. 1b, Table 1 and Supplementary Table 4). On the basis of this finding, we searched for possible tumour-specific mutations of *A20* by genomic DNA sequencing of entire coding exons of the gene in the same series of lymphoma samples (Supplementary Fig. 5). Because two out of the three Hodgkin's-lymphoma-derived cell lines had biallelic *A20* deletions/mutations (Supplementary Fig. 6), 24 primary samples from Hodgkin's lymphoma were also analysed for mutations, where

[1]Cancer Genomics Project, Department of [2]Pediatrics, [3]Cell Therapy and Transplantation Medicine, and [4]Hematology and Oncology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. [5]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, 4-1-8, Honcho, Kawaguchi-shi, Saitama 332-0012, Japan. [6]Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogo-in, Sakyo-ku, Kyoto 60 6-8507, Japan. [7]Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. [8]Division of Pathology, The Cancer Institute of Japanese Foundation for Cancer Research, Japan, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan. [9]Hematology Division, Hospital, and [10]Early Oncogenesis Research Project, Research Institute, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. [11]Division of Immunology, Aichi Cancer Center Research Institute, 1-1 Kanokodeta, Chikusa-ku, Nagoya 464-8681, Japan. [12]Gunma Children's Medical Center, 779 Shimohakoda, Hokkitsu-machi, Shibukawa 377-8577, Japan. [13]Division of Hematology, Internal Medicine, Showa University Fujigaoka Hospital, 1-30, Fujigaoka, Aoba-ku, Yokohama-shi, Kanagawa 227-8501, Japan. [14]Department of Pathology, Tokyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan.

- Genetic analysis for SNPs and deletions in B-cell lymphomas
- Large number of hits on the NF-κB regulator, A20
- Examined the ability of wild-type and cancer-associated A20 mutants to control NF-κB activity.
- Utilized the pGL4.32 [*luc2P*/NF-κB-RE/Hygro] Vector to assay NF-κB activity.

Kato, M., *et al.* (2009) *Nature* **459**, 712-716.

Promega

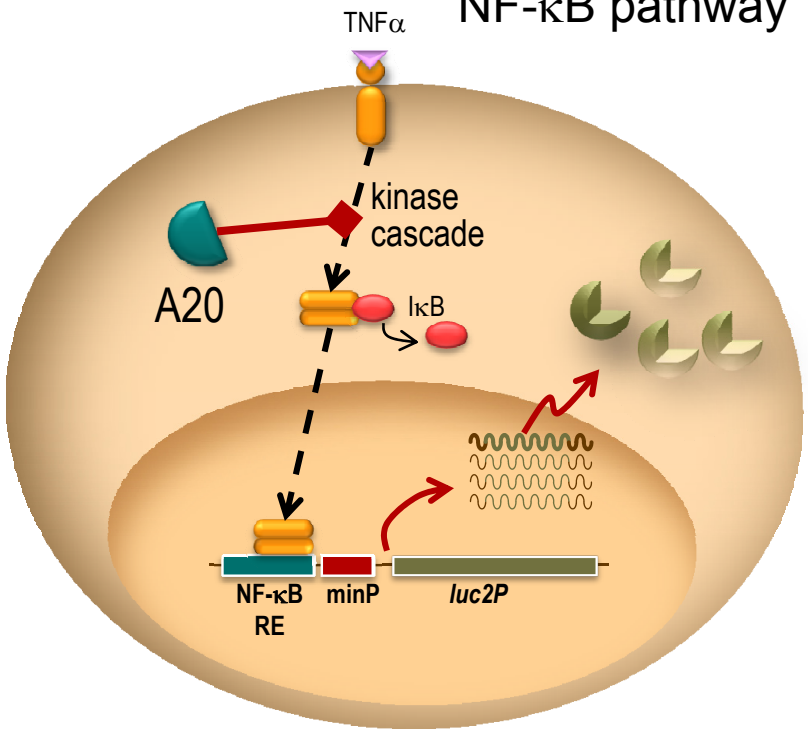
Case Study:
***Kato, M.*, et al.**

A20 expression stops NF-κB pathway

KM-H2 cells do not express A20 (double mutant)

Autocrine activation of NF-κB pathway

A20 expressed through on/off system



A20 on
↓ Luc Activity
↓ Viability
↑ Apoptosis

Lymphoma mutant A20's expression through on/off system

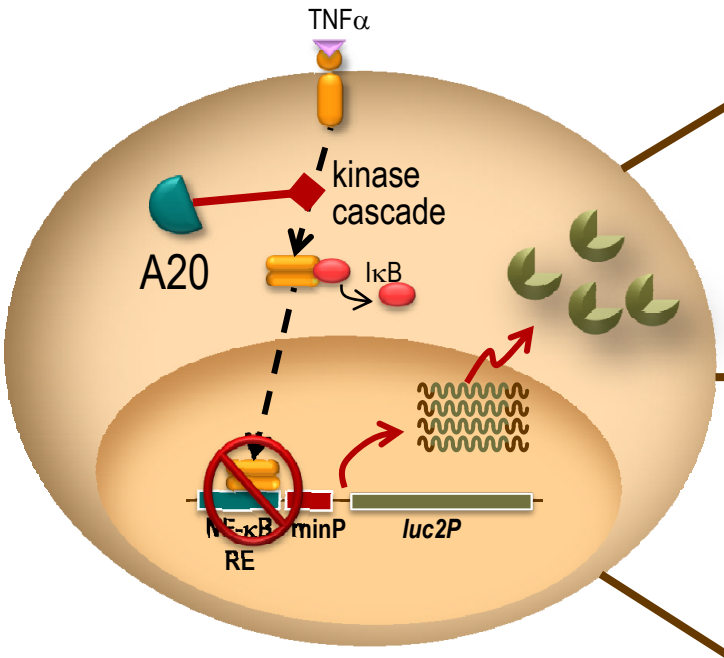
A20 Mutant on
= Luc Activity
↑ Viability
X Apoptosis

KM-H2 lymphoma cells stably transfected with pGL4.32[*luc2P*/NF-κB RE/Hygro] Vector

Promega

Case Study:
***Kato, M.*, et al.**

How the authors determined the cell state



Assay for apoptosis with an Annexin V Assay

10^6 cells/assay
Flow cytometry

Assay for remaining viable cells by measuring reducing potential

96-well assays
Spectrophotometer

Assay for luciferase activity with Luciferase assay requiring production of cell lysate

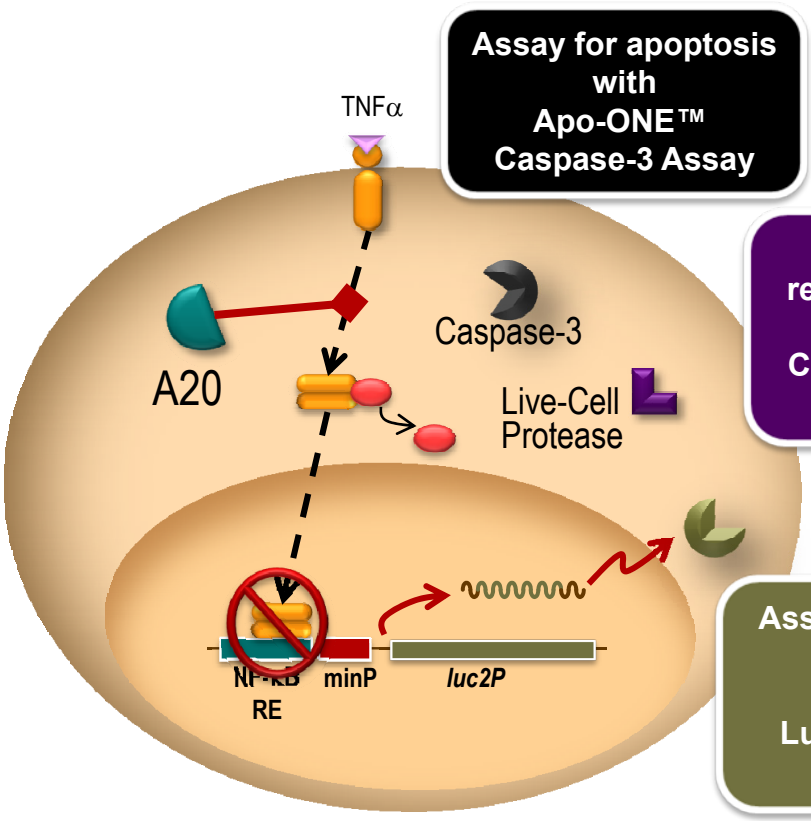
96-well assays
Luminometer

3 Separate Assays
3 Separate sets of cells

Promega

Case Study:
Kato, M., et al.

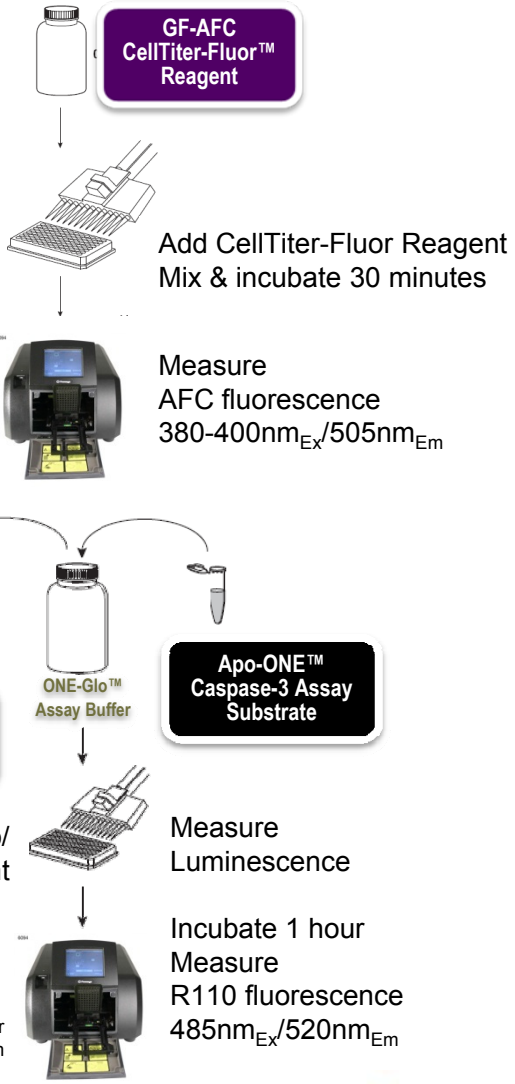
The assays could have been streamlined…



Assay for apoptosis with Apo-ONE™ Caspase-3 Assay

Assay for remaining viable cells with CellTiter-Fluor™ Assay

Assay for luciferase activity with ONE-Glo™ Luciferase Assay System



Measure viable cell number, apoptosis & luciferase activity

- on the same cells
- in the same well
- in the same experiment

Promega

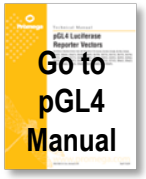
More Information

- pGL4.29 [*luc2P*/CRE/Hygro] Vector Product Protocol
- pGL4.30 [*luc2P*/NFAT-RE/Hygro] Vector Product Protocol
- pGL4.32 [*luc2P*/NF-κB-RE/Hygro] Vector Product Protocol
- pGL4.33 [*luc2P*/SRE/Hygro] Vector Product Protocol
- pGL4.34 [*luc2P*/SRF-RE/Hygro] Vector Product Protocol

- GloResponse™ CRE-*luc2P* HEK293 Cell Line Technical Bulletin
- GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line Technical Bulletin
- GloResponse™ NF-κB-RE-*luc2P* HEK293 Cell Line Technical Bulletin

pGL4.23-pGL4.28 citations from HighWire Press®

pGL4.29-pGL4.34 citations from HighWire Press®



PNotes Schagat, T., Paguio, A. and Kopish, K. (2007) Normalizing genetic reporter assays: Approaches and considerations for increasing consistency and statistical significance. *Cell Notes* **17**, 9-12.

PNotes Paguio, A., *et al.* (2006) Using luciferase reporter assays to screen for GPCR modulators. *Cell Notes* **16**, 22-25.

PNotes Shultz, S., *et al.* (2008) Expanding applications of bioluminescence technology: Predictive bioassays for TNFα biologicals potency and dose-standardization studies. *Promega Notes* **100**, 19-21.

CNotes Schumacher, M., Dicato, M. and Diederich, M. (2008) Improved response ratio for NF-kB inhibition analysis using new luciferase reporter vector (pGL4.32 [*luc2P*/NF-kB-RE/Hygro]). *Cell Notes* **21**, 18-19.

Promega