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TITLE: A Novel Method to Screen for Dominant Negative ATM
Mutations in Familial Breast Cancer

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13. ABSTRACT (Maximum 200 Words) The aim of this proposal is to identify families carrying potentially pathogenic <i>ATM</i> mutations by assaying for <i>ATM</i> kinase activity in cell lines derived from individuals with multiple cases of breast cancer in their family but no pathogenic <i>BRCA1</i> or <i>BRCA2</i> mutation (' <i>BRCAX</i> ' families). If pathogenic mutations in <i>ATM</i> are found in these families it will substantiate the role of <i>ATM</i> in breast cancer susceptibility, allow us to characterise the functional effects of those mutations, and provide clinically valuable information for the families involved. We have analysed 145 cell lines established from index cases from non- <i>BRCA1/2</i> breast cancer families for <i>ATM</i> expression and activity. 10/145 (6.9%) cell lines showed markedly reduced <i>ATM</i> kinase activity, despite normal level of expression of <i>ATM</i> protein. DNA from the index cases with aberrant <i>ATM</i> kinase activity is currently being screened by D-HPLC to determine whether the lack of activity is due to mutations in <i>ATM</i> . Pathogenic <i>ATM</i> mutations (T7271G, IVS10 6T-G) have been constructed in expression vectors. Functional analysis has been performed for V2424G (base T7271G). This mutant form of <i>ATM</i> is quite stable but is intrinsically defective as a kinase. Pilot profiling of wild-type and <i>ATM</i> mutant lymphocyte RNAs after exposure to radiation has established that a large number of signal transduction molecules are dysregulated as a consequence of this mutation.				
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Introduction

The *ATM* gene is mutated in the autosomal recessive disorder, ataxia telangiectasia (A-T), which is characterised by cancer predisposition, cerebellar ataxia and immunodeficiency. One of the most controversial topic in breast cancer genetics is whether mutations in the *ATM* gene predispose women to breast cancer. Studies of A-T families appear to have an elevated frequency of breast cancer in females, particularly in obligate heterozygotes whose risk may be increased as much as 7-fold. By contrast, most studies of sporadic breast cancer have not found an increased frequency of germline *ATM* mutations compared with controls, and linkage analysis of markers close to *ATM* in multiple-case families has provided no evidence that the *ATM* gene predisposes women to breast cancer. Nevertheless, two recurrent *ATM* mutations, T7271G and IVS10-6T->G, were recently reported to be associated with breast cancer (Stankovic *et al.*, 1998; Broeks *et al.*, 2000). We analysed these two pathogenic mutation in *ATM* in female-breast cancer only, non-*BRCA1/2* families in the Australian based Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer (kConFab) and observed that 3% of the families carried one of the two mutations in *ATM* analysed (Chenevix-trench *et al.*, 2002). We have shown that both mutations act as dominant negatives in that *ATM* kinase activity was markedly reduced in the heterozygous carriers of these mutations. These observations suggest that a proportion of hereditary breast cancer may be due to *ATM* mutations, and that the increased breast cancer risk may be restricted to a subset of *ATM* mutations.

The aim of this proposal is to identify families carrying potentially pathogenic *ATM* mutations by assaying for *ATM* kinase activity in cell lines derived from individuals with multiple cases of breast cancer in their family but no pathogenic *BRCA1* or *BRCA2* mutation ('*BRCAX*' families). In addition, we aim to identify target genes dysregulated by *ATM* mutations, and ideally to develop a novel method of high throughput screening for *ATM* mutations.

Our hypotheses were that:

- a) Impaired activation of *ATM* kinase in lymphoblastoid cell lines (LCLs) from index cases of multiple case non-*BRCA1/2* breast cancer families will provide a high-throughput screening method for identifying families carrying dominant negative mutations in *ATM*
- b) Microarray analysis of LCLs from heterozygotes with dominant negative *ATM* mutations, and of stable transfectants generated with mutant *ATM* constructs, will identify a unique set of target genes dysregulated as a consequence of these *ATM* mutations. The information gained can be used to develop an alternate method for high-throughput screening and may also provide candidates for the genes involved in the genesis of *ATM*-related breast tumours.

This project will establish whether impaired activation of *ATM* kinase and/or microarray analysis can be used as powerful tool (s) to identify families carrying dominant negative

mutations in *ATM*. This will have important clinical ramifications for the families involved in terms of pre-symptomatic diagnosis, surveillance and risk management.

Body

Task 1 - Assay for ATM kinase activation in LCLs from the youngest affected person from 260 high-risk breast cancer families without a pathogenic mutation in BRCA1 and BRCA2 (months 1-6).

At our request, kConFab have established EBV-transformed cell lines from index cases (the youngest affected individual from each family) from 200 high-risk breast cancer families without a pathogenic mutation in BRCA1 or BRCA2. We anticipate that an additional 60 LCLs will be available to us within the next 3 months. These LCLs grew slowly in culture and took about 6 months to establish well in-vitro. ATM expression was determined by western blotting with anti-ATM antibody and ATM kinase activity was assayed by immunoprecipitation kinase assays as described by us previously (Gatei *et al*, 2000). The activation was also measured *in vivo* using anti-phospho-specific antibodies against ATM phosphorylation site in p53 (phosphoSer15). Previous work from the laboratory and others have shown that ATM is required only for the immediate and rapid phosphorylation of its targets after ionizing radiation since they are still phosphorylated in ATM-null cells, albeit with delayed kinetics (Khanna *et al*, 1998, Gatei *et al*, 2001). Therefore, the activation of ATM was assayed within 30 mins of exposure to ionizing radiation (6 Gy). 10 out of the 145 cell lines tested showed reduced ATM activity, although the level of expression of ATM was quite comparable to controls (Fig. 1).

Task 2 – Start microarray expression profiling with LCLs from 7 non-carriers and 7 carriers each with the T7271G and IVS10-6T>G mutation, with and without prior exposure to ionising radiation (months 6-12).

This task has been delayed because of the difficulty we had in finding a suitably qualified post-doctoral scientist, given the huge demand world-wide for people with microarray experience. We advertised at the 2002 American Association of Cancer Research meeting but received no applications. We were therefore fortunate to identify a candidate, Nicola Turton, in the UK who was completing her PhD in microarray analysis, and wanted to move to Australia. Nicola completed her PhD in January 2003, and was then able to apply for a visa which she did immediately. She received her visa on March 17th and left the UK the same day, starting work on March 20th.

Since her arrival she familiarised herself with the necessary image analysis (ImaGene5.0, BioDiscovery Inc), data-mining (GeneSpring5.5, Silicon Genetics Inc) and data warehousing tools we have established in house (BASE: <http://microarray.imb.uq.edu.au/BASE>). Nic has also successfully commenced microarray expression-profiling experiments using Compugen 19,000 gene long-oligo

arrays that were fabricated in house. These pilot experiments have compared the following samples in pair-wise experiments:

- 1) wild type Vs T7271G homozygote lymphocytes after exposure to 3 Gy radiation and incubation for 0, 2 and 6hrs.
- 2) wild type Vs T7271G heterozygote lymphocytes after exposure to after exposure to 3Gy radiation and incubation for 0, 2 and 6hrs.

These experiments have established that there are differences in the transcriptional response of wildtype Vs ATM mutant lymphocytes. Functional annotation of these differentially expressed genes revealed a large number of signal transduction molecules as well as genes of no known function.

Task 3 – Engineer mutant constructs and generate transfections with wild-type and mutant constructs. Do microarray analysis with wild-type and mutant transfections, and cell lines from individuals with different ATM and BRCA1/2 genotypes (months 1-18)

We have constructed the pathogenic *ATM* missense mutation (V2424G; T7271G), which confers a high risk of breast cancer, using cDNA mutagenesis. The mutant and wild-type *ATM* cDNAs were also tagged with a Green-Fluorescent Protein at their N-terminus. *ATM*-null cells were transiently transfected with wild-type and mutant *ATM* cDNA constructs and *ATM* expression and kinase activity was determined with or without prior exposure of cells to 6 Gy irradiation. Kinase activity was performed in an *in vitro* assay using p53 as a substrate. When compared with wild-type *ATM*, the mutant *ATM* had no activity. Failure to observe kinase activity was not caused by lack of expression or instability of mutant protein, because immunoprecipitation with anti-*ATM* antibodies showed that the mutant protein was expressed at levels equal to wild-type *ATM* (Fig. 2). To confirm our observations, we performed an *in vivo* kinase assay by immunofluorescence using a phospho-Ser/Thr, *ATM*/*ATR* substrate antibody, raised against a collection of peptides containing *ATM* consensus motif; serine or threonine followed by glutamine (SQ/TQ). *ATM*-deficient cells were grown on coverslips to 50% confluence, then transiently transfected with plasmids expressing wild-type *ATM* and the mutant *ATM*. At 16 hrs post-transfection cells were subjected to 6 Gy of irradiation and incubated for 30 mins under normal growth conditions prior to staining with anti-phospho-(Ser/Thr) *ATM*/*ATR* substrate antibody. Irradiated cells which expressed wild-type *ATM* showed a strong staining for phosphorylated *ATM* substrates, whereas un-irradiated cells displayed only a background level of staining (Fig. 3). Cells expressing mutant form of *ATM* failed to elicit kinase activity and displayed only background level of staining for phosphorylated *ATM* targets with or without irradiation (Fig. 3). The results clearly indicate that the mutant form of *ATM* is quite stable but is intrinsically defective as a kinase. We were unsuccessful in generating cell lines that stably express the mutant form of *ATM* cDNA. Therefore, this pathogenic mutation in *ATM* has also been constructed in EBV-based episomal vector, pMEP4, under the control of metallothionein II-inducible promoter. This mutant cDNA has been transfected into LCLs obtained from two normal and two AT individuals. Control and AT cell lines

stably expressing the mutant form of ATM have been selected (Fig. 4) and are currently being analysed biochemically and phenotypically for various aspects of ATM function.

The minigene with IVS 10 6T-G has also been constructed and we are currently performing similar functional analysis with this mutant.

Task 4 – Extend the analysis of ATM kinase activity to all available family members based on results with the index cases (months 13-18).

The LCLs from the first 5 BRCAx families we identified with aberrant ATM kinase activity were requested from kConFab in February and the first of these have been established and are due to arrive in the lab on 29th April. The remainder will be requested when the screening of all 260 LCLs has been completed.

Task 5 – Start mutation analysis of the ATM gene in families with compromised ATM kinase activity (months 15-24).

DNA from the first 5 index cases found to have aberrant ATM kinase activity are currently being screened by D-HPLC to determine whether the lack of activity is due to mutations in ATM.

Key Research Accomplishments

- 1 200 LCLs have been established from index cases from non-BRCA1/2 breast cancer families.
- 2 145 LCLs have been analysed for ATM expression and activity.
- 3 10/145 (6.9%) LCLs showed markedly reduced ATM kinase activity, despite normal level of expression of ATM protein
- 4 Pathogenic ATM mutations (T7271G, IVS10 6T-G) have been constructed in expression vectors. Functional analysis has been performed for T7271G. This mutant form of ATM is quite stable but is intrinsically defective as a kinase.
- 5 Establishment of Microarray informatics (annotation, datamining, warehousing) and pilot profiling of wild-type Vs ATM mutant lymphocyte RNAs after exposure to radiation.

Reportable outcomes

- Presented at the Australian Health and Medical Research Congress, Melbourne, November 25th-29th 2002
- Presented at Ataxia-telangiectasia and ATM 2002 International Symposium, Boston Massachusetts- May 2002
- Presented at the Queensland Molecular Biology Meeting, New Zealand, August 2002

- Presented at the 'The Epidemiology of the ATM gene', Lillehammer, Norway, June 2002
- Presented at the Lorne Cancer Conference, February 2003
- 200 lymphoblastoid cell lines have been established from index cases of multiple case-breast cancer families with no pathogenic mutations in BRCA1/BRCA2. These are available to other researchers through kConFab (<http://www.kconfab.org>).
- An Excel database has been established to record receipt of LCLs, and the results of assays of ATM function.

Conclusions

10/145 (6.9%) lymphoblastoid cell lines from index cases of multiple-case breast cancer families have been identified with markedly reduced ATM kinase activity suggesting that these cell lines might contain pathogenic mutations in ATM. If pathogenic mutations in *ATM* are found in these families it will substantiate the role of *ATM* in breast cancer susceptibility, allow us to characterise the functional effects of those mutations, and provide clinically valuable information for the families involved.

References

Broeks A, Urbanus JH, Floore AN, et al: ATM-heterozygous germline mutations contribute to breast cancer- susceptibility. *Am J Hum Genet* 66:494-500., 2000

Chenevix-Trench G, Spurdle AB, Gatei M, et al. Dominant negative ATM mutations in breast cancer families. *J Natl Cancer Inst.* 94:205-15., 2002.

Gatei M, Scott SP, Filippovitch I, et al: Role for ATM in DNA damage-induced phosphorylation of BRCA1. *Cancer Res* 60:3299-304., 2000

Gatei, M, Zhou, BB, Karen H, et al: ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of Brca1 at distinct and overlapping sites. In vivo assessment using phospho-specific antibodies. *J Biol Chem.* 276:17276-80, 2001.

Khanna KK, Keating KE, Kozlov S, et al: ATM associates with and phosphorylates p53: mapping the region of interaction. *Nat Genet* 20:398-400., 1998

Stankovic T, Kidd AM, Sutcliffe A, et al: ATM mutations and phenotypes in ataxia-telangiectasia families in the British Isles: expression of mutant ATM and the risk of leukemia, lymphoma, and breast cancer. *Am J Hum Genet* 62:334-45., 1998

Figure legends

Fig.1 Identification of lymphoblastoid cell lines (LCLs) with reduced ATM kinase activity. Cells were harvested 30 min after exposure to 6 Gy of ionizing radiation (IR). Whole cell extracts were prepared and equal amount of proteins were run on SDS-PAGE gels and western blotted with anti-phosphoserine-15 antibody for *in vivo* assessment of ATM kinase activity and anti-ATM antibody to determine the level of expression of ATM protein. Western blotting with anti-DNA-dependent protein kinase was used as a control for equal protein loading. C3ABR, wild-type ATM expressing cell line is used as positive control for ATM activity and L3, ATM-deficient cell line, is used as negative control. The first 5 LCLs identified with reduced ATM kinase activity are shown along with 2 LCLs with activity comparable to the positive control LCL.

Fig.2 Functional analysis of V2424G (base T7271G) mutant ATM protein. HEK-293T cells were transiently transfected with a GFP vector alone, GFP-wt ATM and GFP-mutant ATM. Whole cell extracts were prepared and immunoprecipitated with anti-GFP antibody (Panel A). *In vitro* kinase assays were performed on immunoprecipitated ATM using GST-p53 (aa1-40) as a substrate and $\gamma^{32}\text{P}$ ATP. Samples were analysed by SDS-PAGE, followed by immunoblotting with anti-GFP antibody to determine protein expression (Panel A) and autoradiography to measure incorporation of phosphate groups into p53 (Panel B). *In vivo* activity was assessed by immunoblotting with anti-phosphoserine-15 antibody (Panel C). The cell extracts were prepared with (+) or without (-) prior exposure of cells to 6 Gy ionizing radiation (IR).

Fig.3 *In vivo* kinase activity of GFP-wild-type and GFP-V2424G mutant ATM against multiple ATM targets. ATM-deficient cell line transiently expressing GFP-wild-type ATM, GFP-V2424G mutant ATM protein were subjected to 6 Gy of ionizing radiation and harvested 30 min after for immunostaining with phospho-serine ATM substrate antibody, raised against collection of peptides containing ATM consensus motif. GFP-ATM (Green) is shown to phosphorylate ATM substrates (red) in response to DNA damage.

Fig. 4 Stable transfection of control (C3ABR) and AT (AT3ABR) cell line with mutant form of ATM (V2424G). C3ABR and AT3ABR cell lines were transfected with V2424G mutant ATM protein, and stable cell lines were established by selection in hygromycin. Expression of mutant ATM protein was induced with cadmium chloride for 6 h before irradiation (6 Gy IR) and incubation for 30 min. ATM expression was determined by immunoprecipitation with anti-flag followed by immunoblotting with same antibody.

Fig.1

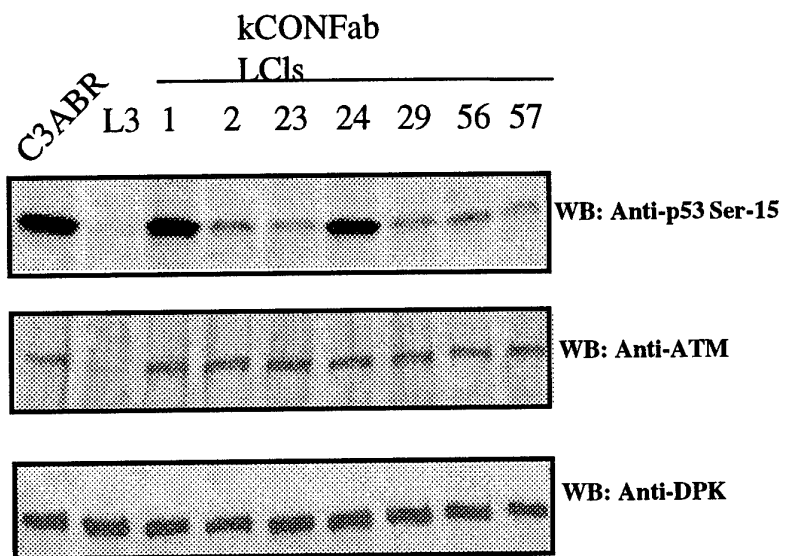


Fig. 2

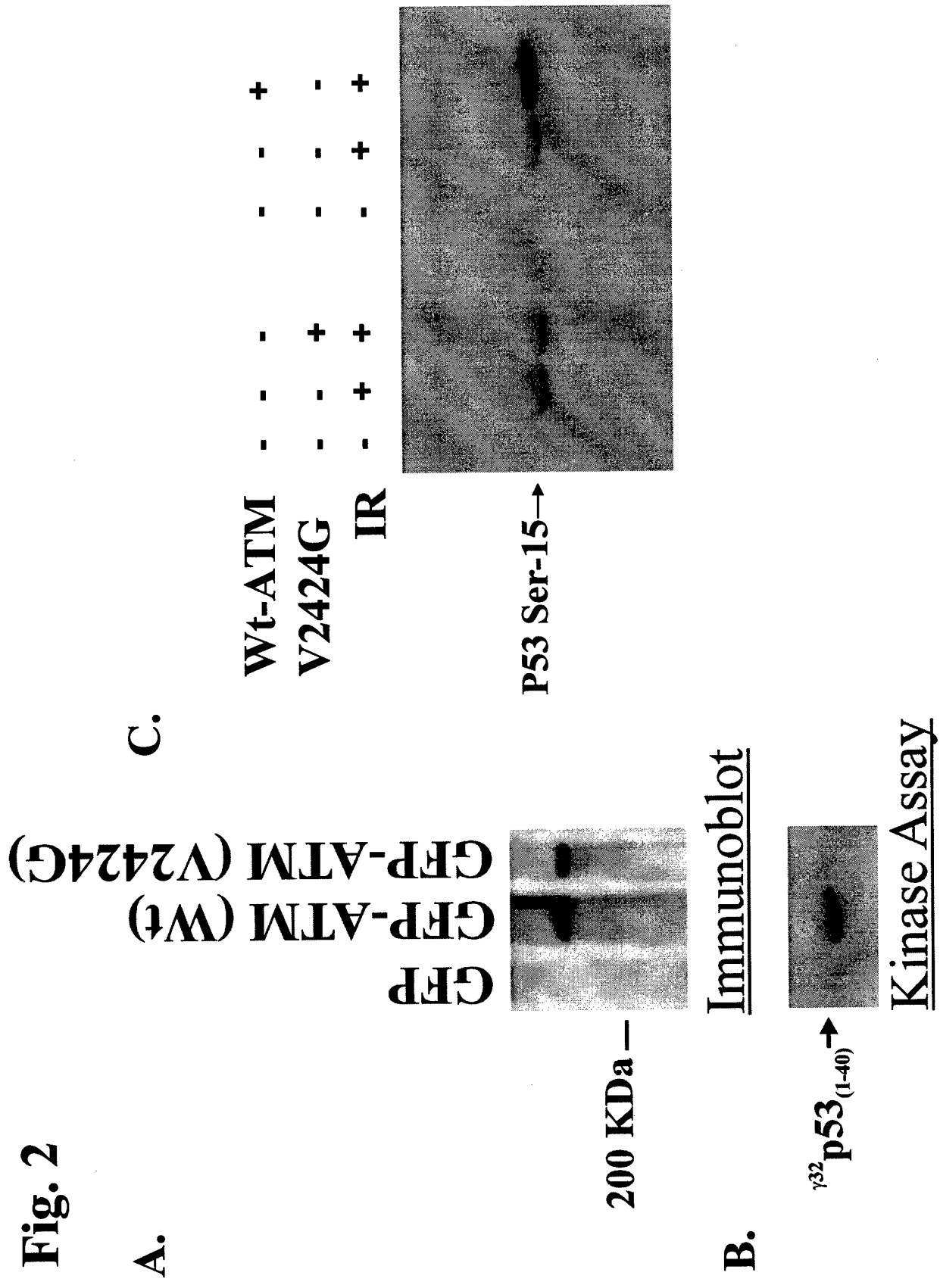


Fig. 3

GFP-ATM Phos Ser/Thr DNA IR

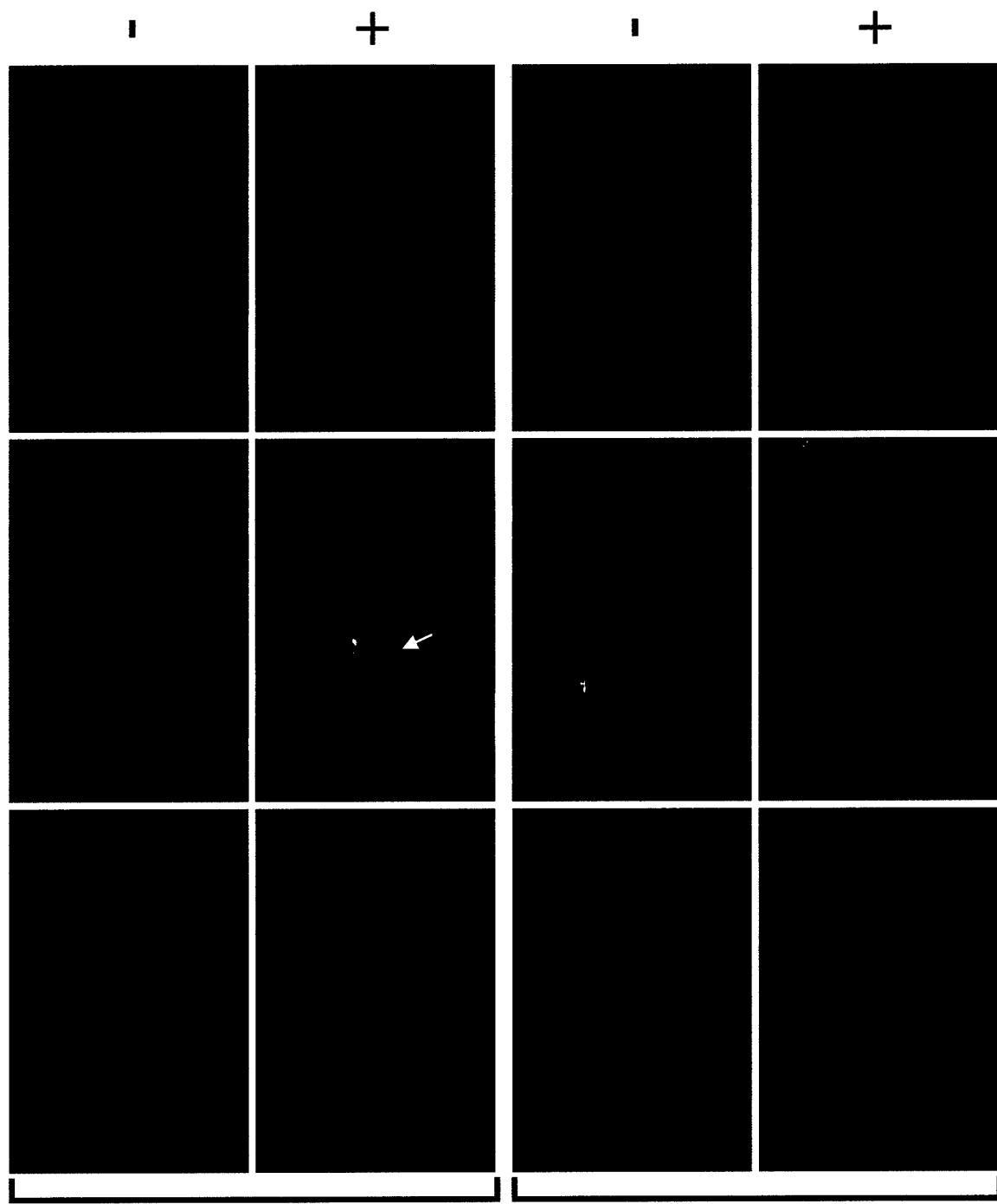
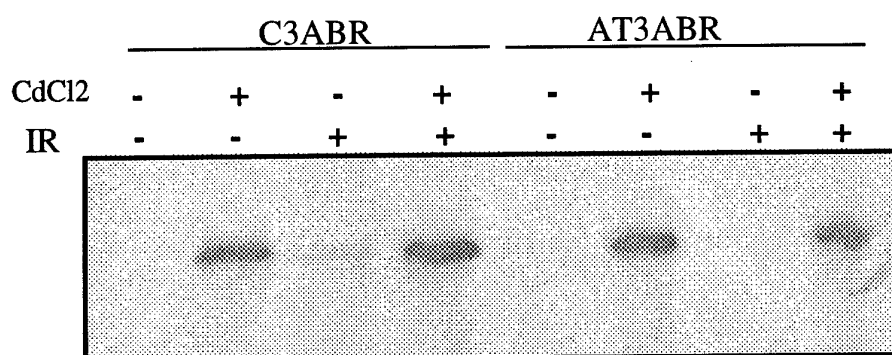


Fig.4



IP: anti-flag
WB: anti-flag