行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

計畫名稱: Pir51 在 DNA 修補途徑的功能性探討

計畫類別：■個別型計畫　□整合型計畫

計畫編號：NSC　96－2320－B－039－034－MY3

執行期間：　96 年　08 月　01 日至　99 年 07 月　31 日

執行機構及系所：中國醫藥大學 生物科技學系

計畫主持人：蔡士彰

共同主持人：

計畫參與人員：陳柏翰、黃聖峰、陳東廷、陳加恩、王建喬、曾沛琪

成果報告類型(依經費核定清單規定繳交)：□精簡報告　■完整報告

本計畫除繳交成果報告外，另須繳交以下出國心得報告：

□赴國外出差或研習心得報告

□赴大陸地區出差或研習心得報告

■出席國際學術會議心得報告

□國際合作研究計畫國外研究報告

處理方式：除列管計畫及下列情形者外，得立即公開查詢

□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

中　華　民　國　99 年　10 月　31 日

	頁數
目錄	II
1. 中、英文摘要及關鍵詞 (keywords)	III
2. 報告內容	
1.1 前言	1
1.2 研究目的	1
1.3 文獻探討	2
1.4 研究方法	3
1.5 結果與討論	6
2. 參考文獻	17
3. 計劃成果自評	18

計畫中文摘要

Pir51 是一個存在於 Rad51 複合體，但功能未知的蛋白質。我們之前顯示，Pir51 和 Rad51 表示幾乎相同地被調控在細胞週期期間。然而，Pir51 只有少許功能在一個需要 Rad51 的同源結合修復過程。 這提高 Pir51 促進癌症的可能性，正如同它配對的夥伴 Rad51 的情況。我的提案著重於探討 Pir51 在 DNA 破壞和修復的生物性功能。我的提案提供 Pir51 蛋白質在 DNA 破壞和修復控制的新洞察方向。第一年具體目標: 做 Pir51 剔除細胞株。我使用二種方法探討 Pir51 蛋白質的功能。 一種是功能喪失策略,另一種是功能獲取策略。在功能喪失策略, siPir51 表達質體已被構建和測試調控 Pir51 蛋白質表達。另一方法使用功能獲取策略是構建誘導標記的 Pir51 蛋白質表達的細胞株。它提供無價的洞察 Pir51 蛋白質在 DNA 修復途徑的分子機制。第二年具體目標: 鑑定與 Pir51 結合蛋白質。Rad51 已被證明與很多蛋白質結合包括 RPA 、BRCA2 和 ATM。因為 Pir51 與 Rad51 結合, 我們使用免疫沉澱法去辨認 Pir51 複合體。我們獲得很多資訊從 Pir51 結合的蛋白質並將我們探索 Pir51 的不同生物角色。第三年具體目標: 描繪 Pir51 蛋白質的穩定性和確定 Pir51 缺陷在 DNA 受損的反應。我們使用 ubiqitination 檢視 Pir51 蛋白質的穩定性。這些研究提供對在 DNA 受損和修復控制的複雜機制之詳盡的理解。

關鍵詞: **DNA 修復 ，細胞週期，和泛素化**

計畫英文摘要

Pir51 is a protein of previously unknown function that exists in a complex with Rad51. We showed that Pir51 and Rad51 expression are almost identically regulated during the cell cycle. Pir51, however, has surprisingly little effect on homologous recombination repair, a process for which Rad51 is essential. Taken together, it raises the possibility that over-expression of Pir51 may promote cancer, as is the case for its pairing partner, Rad51. My proposal provides essential new insights for the Pir51 protein in the control of DNA damage and repair. **SPECIFIC AIM 1: Make a *Pir51* knockout RNAi cell line.** I used two approaches to characterize functions of the Pir51 protein. One is the loss of function strategy, and the other is the gain of function strategy. In the loss of function method, an siPir51 expression plasmid has been constructed and tested for down-regulating the protein level of Pir51. The other approach is using the gain of function strategy to make a constitutive or/ and an inducible cell line expressing the FLAG-tagged Pir51 protein. It provides invaluable insights into the molecular mechanisms of the Pir51 protein in DNA repair pathways. **SPECIFIC AIM 2: Identification of proteins that interact with Pir51.** Rad51 has been reported to biochemically interact with a large number of proteins including RPA, BRCA2, and ATM. Since Pir51 binds to Rad51, we will immunoprecipitate and identify the Pir51 complex. We obtain a lot of information from Pir51 interacting proteins and explore different roles of Pir51. **SPECIFIC AIM 3: Characterize the protein stability of Pir51 and determine the defects of Pir51 in the DNA damage response.** We examine the protein stability of Pir51 by ubiqitination. These studies ultimately provide essential new insight for a thorough understanding of the intricate mechanisms in the control of DNA damage and repair.

Keywords: DNA repair, cell cycle, and ubiqitination

1. 報告內容

1.1 前言:

本計劃為探討 Pir51 (protein interacting with Rad51)在細胞內的功能。先前我們發現與 indolent small lymphocytic lymphoma (SLL)比較後，Pir51 在 aggressive mantle cell lymphoma (MCL)被高度表達。再者，我們發現 Pir51 調控 DNA crosslink such as MMC (Mitomycin C)修補卻對同源性重組修補(homologous recombination repair, HRR)沒有顯著的影響。此現象非常類似 the Fanconi Anemia (FA) proteins 結合 Rad51 所參與修補 DNA crosslinks 機制。 BRCA2 (後來發現與 FancD1 是同一基因)為 Fanconi Anemia (FA) proteins 家族的其中成員之一，且 BRCA2 and BRCA1 突變已知為遺傳性 breast or ovarian cancers 主要原因。BRCA2 與 RAD51 在 DNA 遭受破壞時會形成 foci. 同時，BRCA2 也被認為與維持基因組完整性(genome integrity)有相當大的關連性。

藉由生物化學的方法與遺傳的方法探討 Pir51 在細胞內的功能，可以使我們更清楚同源性重組修補機制，並增加對化學藥劑(例如 Mitomycin C)在化學療法的了解，以利日後治療發展。

1.2 研究目的:

壹、 The understanding of the biological functions of Pir51 in cell cycle, homologous recombination, DNA replication, and DNA repair pathways.

貳、 建立起 pir51 與基因不穩定性(genome instability)的相關機轉。 The Fanconi anemia (FA) proteins are parsed into more than 11 complementation groups that control genome integrity, with numerous papers focusing on this group of proteins and the biological processes they control. The FA proteins include some of the most intensively studied DNA repair and cancer causing genes in biology. Our studies will place Pir51 into its appropriate context as a regulator of DNA repair and genome stability.

參、 The establishment of connections between biochemical defects of Pir51 and clinical diseases

肆、 了解 DNA repair 的機轉，以利於日後治療發展 (therapeutic intervention)。 Essentially, Pir51 is a newly identified regulator of DNA crosslink repair and a controller of genome integrity. Since cancer is a disease of genome instability, Pir51 has a central role in controlling malignant transformation. By defining how Pir51 performs surveillance of the genome to inhibit malignancy when it is expressed at appropriate levels, a new understanding of DNA repair and possibly novel target proteins and pathways for therapeutic intervention will be revealed.

1.3 文獻探討:

一 探討 PIR51 在 DNA repair pathways 所扮演的角色:

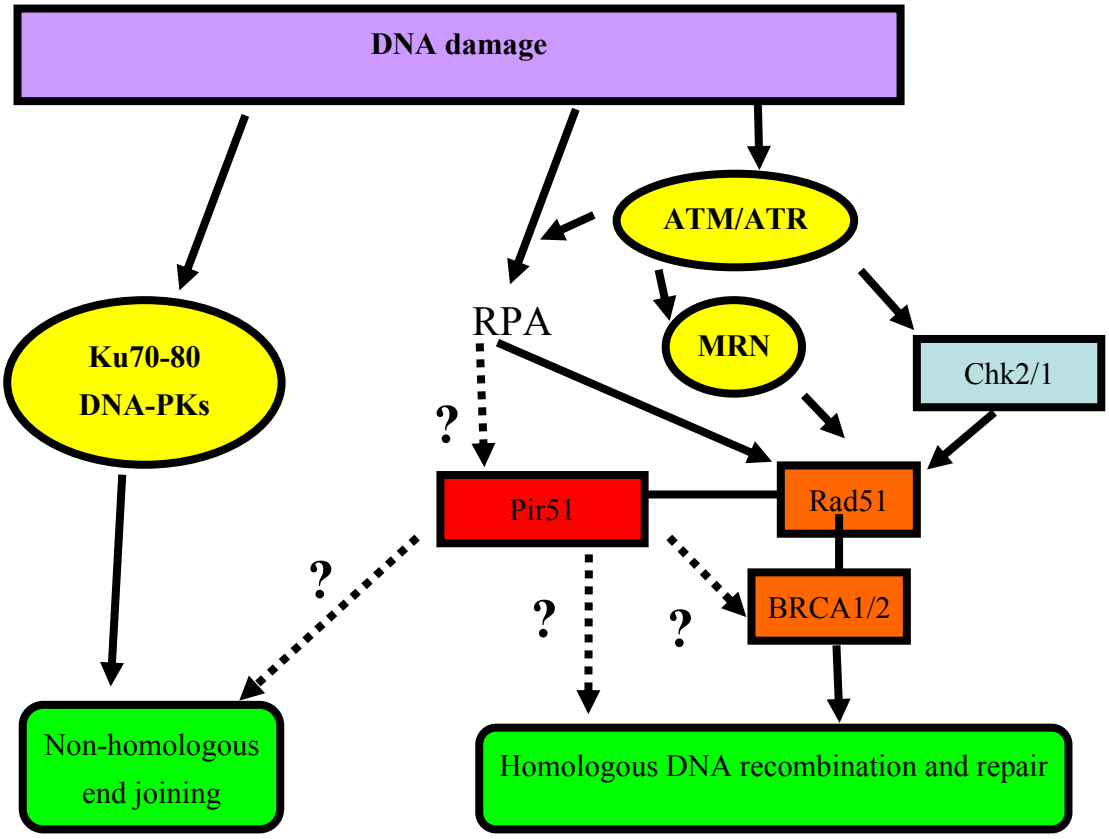


Figure 1: PIR51 可能在 DNA repair pathways 所扮演的角色. There are two major DSB repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR). DSBs induced by radiation and UV light are preferentially repaired by NHEJ in mammalian cells. On the other hand, DSBs resulting from blocked replication are repaired primarily by HR. The five Rad51 paralogs, which include XRCC3, appear to act as a functional unit to promote Rad51 assembly at DSB sites. Pir51 與 Rad51 有相互結合作用，因此 Pir51 可能有 Rad51-dependent pathway and Rad51-independent pathway.

二 探討 PIR51 在 tumorigenesis 所扮演的角色與藥物治療

Pir51 在癌細胞表現量情形

我們發現與 indolent small lymphocytic lymphoma (SLL)比較後，Pir51 在 aggressive mantle cell lymphoma (MCL)被高度表達，而 Pir51 被發現在肝癌細胞表現量下降。

Pir51-deficiency causes chromosome breaks in MMC-treated cells

Treatment of HeLa cells with MMC alone, or with siRNA directed against Pir51 alone, did not increase the frequency of chromosome breaks in metaphase chromosome spreads over untreated cells. MMC 不會影響 Pir51 表現量，而 Hydroxylurea(一種藥物使細胞停留在細胞週期的 S 期)會增加 Pir51 表表達。

1.4 研究方法:

To make a constitutive cell line expressing the FLAG-tagged Pir51 protein and characterize the Pir51 protein complex:

To understand the roles of Pir51, we made a stable cell line expressing. Meanwhile, we made a peptide antibody specific for the amino terminus of Pir51. We performed one dimensional (figure 2) and two dimensional (figure 3) gels to characterize the pir51 protein complex.

Determine whether Pir51 interacts with BRCA2 and other proteins.

To examine whether Pir51 interacts with BRCA2, we used the immunostaining assays to check if Pir51 and BRCA2 colocalize (figure 4). Meanwhile, we also examined whether Pir51 interacts with RPA (replication protein A) (figure 5). In addition, we did co-IP (immunoprecipitation). We used anti-Pir51 antibody to immunoprecipitate the Pir51 protein complex and analyzed the protein interaction with western blotting (figure 6).

Identifying proteins that interact with Pir51

We performed two approaches to isolate and identify Pir51 associated proteins. One of experimental designs is using Matrix Assisted Laser Desorption Ionisation (MALDI)-Time of Flight (TOF) mass spectrometry. The other method is analyzing by screening HeLa cDNA library via the bacteriomatch system. This screen system is similar to the yeast two-hybrid system, but is a fast and reliable system. After the fourth round screen, the positive clones were sequenced. DNA sequences were blasted via NCBI. Positive clones were categorized on the basis of protein functions.

Characterizing the interacting proteins of the Pir51 protein:

We performed the immunoprecipitation method to demonstrate interactions between endogenous interacting proteins and Pir51. In addition, we did immunostaining to show endogenous interacting proteins and Pir51 co-localized together. After double-confirmation, we characterize the biological functions of interacting proteins and Pir51.

Determine if the Pir51 protein co-localizes with other DNA repair proteins:

We performed the immunoprecipitation method to demonstrate interactions between endogenous interacting proteins (PCNA , ATR, and RPA70).

Characterize gene expression profiling in the absence or presence of Pir51 by the cDNA microarray hybridization

We applied the microarray to characterize genes regulated by Pir51. RNA samples were extracted from *Pir51* knockdown cells and control cells. We analyzed three major categories of genes associated with Pir51 specifically in DNA repair pathways, DNA replication, and cell cycle.

To determine the defects of Pir51 in the DNA damage response

細胞培養

將人類子宮頸癌細胞株(HeLa)培養在含有 10% FBS(fetal bovine serum)和 1% P/S(penicillin-streptomycin)的 DMEM(Dulbecco modified Eagle medium)中，放置於攝氏 37 度，5% CO_2 的培養箱培養，每兩天以 trypsinization 方式繼代培養。

抽取 RNA(RNA extraction)

先噴灑 Rnase free 在所要抽取 RNA 的環境。加入 1mL 的 TRIZOL 至離心管中的細胞，放置室溫 5 分鐘，再加入 0.2mL 的氯仿(chloroform)，上下搖晃約 15 秒並且放置室溫 2-3 分鐘。以 12000rpm 、4℃ 的狀況下離心 15 分鐘。離心後，小心的吸取上層 RNA 至新的微量離心管。加入 0.5mL 的異丙醇(isopropanol)，放置冰上 10 分鐘反應。再以 12000rpm 、 4℃ 的狀況下離心 10 分鐘，會看到 RNA 的沉澱物附著在管壁上。將上清液抽掉，並以 75%的乙醇清洗一次後，抽掉上清液，並且以陰乾的方法蒸發多餘的酒精，再以 30ul 含 DEPC 的水回溶。

MetaCore™軟體分析相關路徑

利用 MetaCore™軟體，將人類全基因表現晶片結果輸入後，便可分析基因相互作用關聯網絡、各種轉錄因子、生物訊息傳導和代謝反應路徑以及各種生物活性分子的資料。

西方墨點法(Western blotting)

將 HeLa 細胞從培養箱中取出，低速離心去除上清液之後，再用 0.1% NP40+PBS 的 lysis buffer 將細胞打散並且低速離心，上清液定量後分別加入 loading dye 放乾浴器 95˚C 煮 5 分鐘。(膠體置備，配置 10%的 acrylamide gel:取 5ml 的 next gel buffer，加入 50ul 的 Ammonium persulfate(APS)，5ul 的 TEMED。以 100V，2.5 小時將蛋白質依分子量大小分離。分離蛋白質後，膠體用 200mA，轉漬 2 小時至 PVDF membrane 上。取出 PVDF membrane 後，浸到 5%的牛奶(用 PBST 溶)做 blocking，室溫下搖晃 1 小時。將 PVDF membrane 從牛奶中取出，放入含有一抗的 PBST 中(1:1000)，4℃ 隔夜搖晃。隔天取出 PVDF membrane，以 PBST 清洗，每搖晃 5 分鐘換新的 PBST，重複 3 次。之後取出 PVDF membrane，放入含有二抗的 PBST 中(1:7000)，室溫下搖晃 1 小時。取出 PVDF membrane，以 PBST 清洗，每搖晃 5 分鐘換新的 PBST，重複 3 次。最後使用化學冷光呈色劑與底片壓片偵測蛋白質的表現，並用 Adobe Photoshop 軟體分析量化結果。

冷光報導基因表現分析(luciferase assay)

將 target pir51 之 shRNA 及 pISRE-luciferase (IFN-stimulated response element, ISRE)轉染至 6-well 培養盤中 $3X10^5$ HeLa 細胞，48 小時後，收集並溶裂細胞，加從 Promega 購得 LAR II (Luciferase Assay Subtrate in Luciferase Assay Buffer II)，使用 SpectraMax L 冷光儀去偵測 firefly luciferase activity 和 firefly luciferase activity。

反轉錄酶-聚合連鎖反應(Reverse Transcription-PCR)

參照FastStart Universal Probe Master Protocol(ROX)。在預測的RNA sample內加入特定的引子(primer)，以65℃ 10分鐘反應，來denature RNA的二級結構，再加入RT-master mix以55 ℃ 30分鐘、85℃ 5分鐘反應。

即時定量聚合酶連鎖反應(real-time PCR)

參照 FastStart Universal Probe Master Protocol(ROX)。將已反轉錄的互補 DNA(cDNA)、 master mix、特定的引子及 Nuclease-free water 配成一管試劑，再置入 96 Detection Plate 中，封上封膜後使用 ABI PRISM 7900 機器去進行即時定量聚合酶連鎖反應--- 95℃Denature，15 秒、Anneal/Exten 60 ℃，1 分鐘、重複 40 個 cycles，將資料進行分析比對。

1.5 結果與討論:

To make a constitutive cell line expressing the FLAG-tagged Pir51 protein: We obtain a stably expressing Flag-pir51 cell line and also make a peptide antibody against Pir51. We performed immunoprecipitation and then ran the SDS-PAGE to analyze the Pir51 protein complex (figure 2). Associated proteins are analyzing by mass spectrometry.

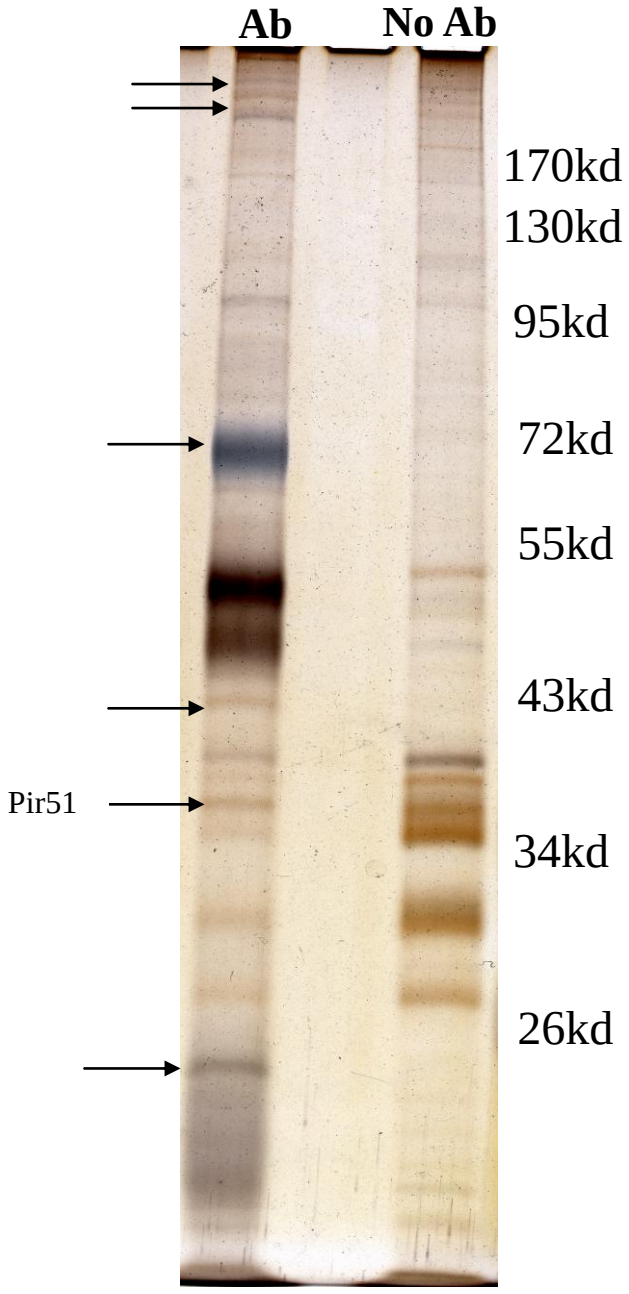
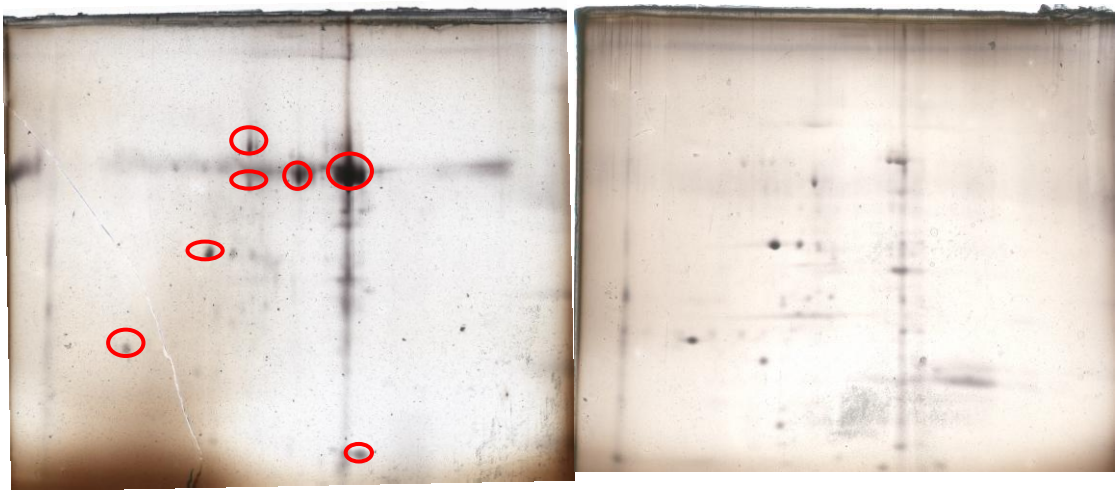


Figure 2 the pir51 protein complex. We extracted proteins from HeLa cells. Nuclear extract fraction was incubated with or without Pir51 antibody for 2 h. After washed, samples were applied into the SDS-PAGE. Gel was stained with a silver staining kit (Bio-Rad). Compared with the no antibody control lane, the specific bands are shown as arrows indicate. Specific bands is analyzing with mass spectrometry.

In addition, we did two dimensional gels to further characterize the Pir51 protein complex (figure 3). So far, as shown in figure 3, the specific spots were isolated and separated. We analyzed what proteins they are.

Figure 3 characterization of the pir51 protein complex using two dimensional gel. We chose wide pH range (pH 3-10) stripes from Bio-Rad biocompany to analyze the difference. Red circles indicate the specific spots were detected only in the pir51 protein complex.

Immunoprecipated with Pir51Ab no Ab



Determine whether Pir51 interacts with BRCA2 and other proteins.

Indeed, we found pir51 co-localizes with BRCA2. As we showed previously in preliminary data, Pir51 interacts with endogenous BRCA2 (figure 4).

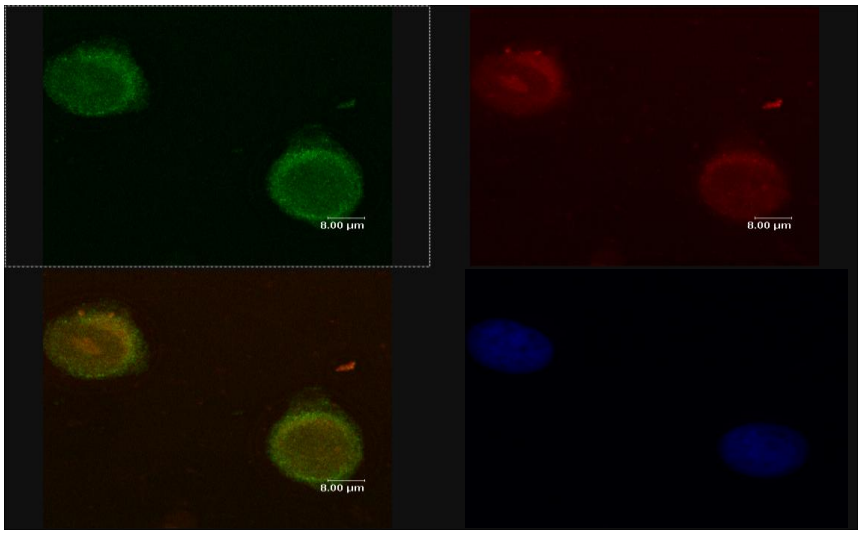


Figure 4 Pir51 co-localizes with BRCA2. We performed the immunostaining assay and found Pir51 co-localizes with BRCA2. Red: Pir51, Green: BRCA2, Blue: DAPI.

Pir51 can be detected in the higher expression level in the S phase during cell cycle. Therefore, we examine whether pir51 interacts with replication protein A (RPA). Surprisingly, RPA32 interacts with and co-localizes with Pir51 (figure 5 and 6). Since there are three members (RPA70, RPA32, and RPA14) in the RPA family, we are detecting whether pir51 interacts with and co-localizes with RPA70 and RPA14.

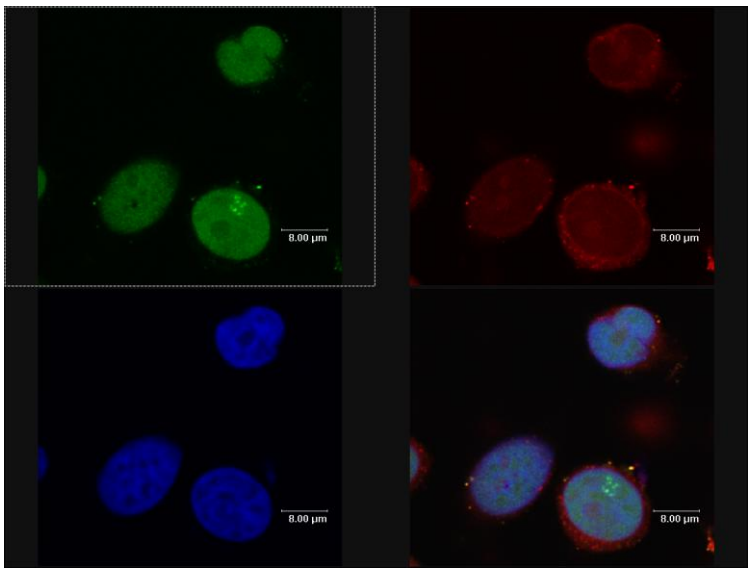


Figure 5 Pir51 co-localizes with RPA32. We performed the immunostaining assay and found Pir51 co-localizes with RPA32. Red: Pir51, Green: RPA32, Blue: DAPI

Figure 6 Pir51 interacts with RPA32. We performed the co-IP assay and found RPA32 in the Pir51 protein complex. Lane 1: no antibody, Lane 2: IP with anti-pir51 antibody, Lane 3: 2% HeLa lysate imput.

Determine whether Pir51 interacts with PCNA and other proteins

Indeed, we found pir51 co-localizes with PCNA (figure 6). Meanwhile, we examined whether pir51 co-localizes with ATR. Surprisingly, we did find pir51 co-localizes with ATR (figure 7).

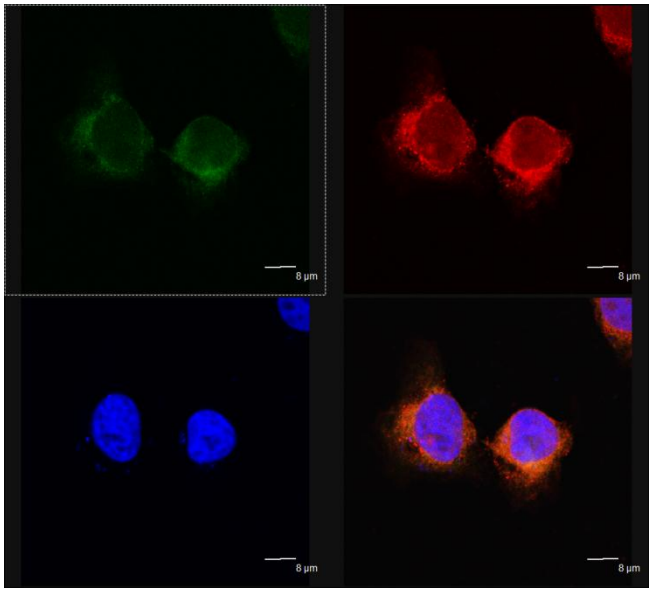


Figure 6. Pir51 co-localizes with PCNA. We performed the immunostaining assay and found Pir51 co-localizes with PCNA. Red: Pir51, Green: BRCA2, Blue: DAPI.

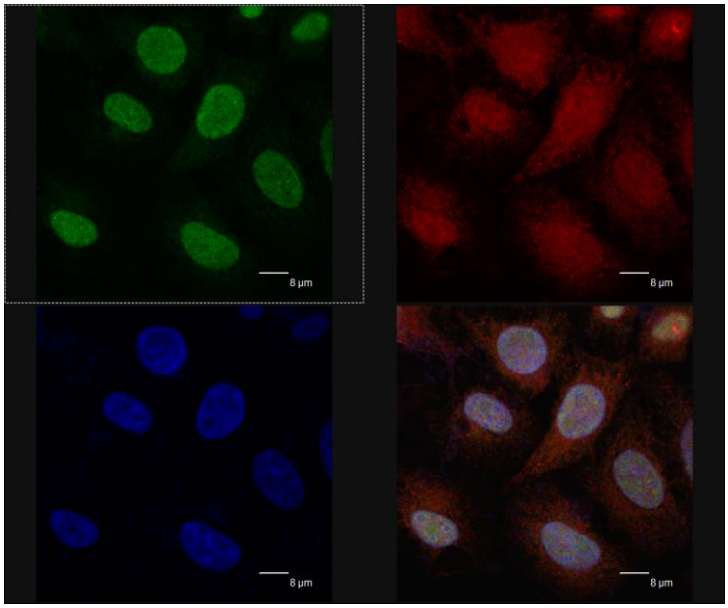


Figure 7. Pir51 co-localizes with ATR. We performed the immunostaining assay and found Pir51 co-localizes with ATR. Red: Pir51, Green: ATR, Blue: DAPI.

Pir51 can be detected in the higher expression level in the S phase during cell cycle. Therefore, we examine whether pir51 interacts with replication protein A (RPA). Surprisingly, RPA32 interacts with and co-localizes with Pir51. Since there are three members (RPA70, RPA32, and RPA14) in the RPA family, we detected whether pir51 interacts with and co-localizes with RPA70 and RPA14. Indeed, we found Pir51 co-localized with RPA70 (figure 8).

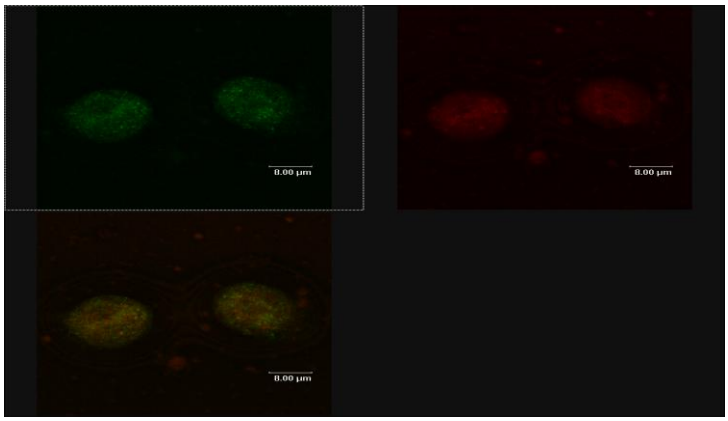


Figure 8. Pir51 co-localizes with RPA70. We performed the immunostaining assay and found Pir51 co-localizes with RPA70. Red: Pir51, Green: RPA70.

Characterize gene expression profiling in the absence or presence of Pir51 by the cDNA microarray hybridization

We checked the protein expression level of the Pir51 knockdown cells (figure 9) and chose clone 2 to further gene expression microarray assays. We are analyzing the data.

Figure 9. The protein expression level of the Pir51 knockdown cells. Proteins were separated and analyzed using western blotting. Lane 1: HeLa lysate, lane 2, 3, and 4: the Pir51 knockdown cells.

Identifying proteins that interact with Pir51:

We performed the bacteriomatch system by screening a HeLa cDNA library. Total 57,371clones were screened, and there are initially 345 positive clones. After the forth round screening, we obtained 147 positive clones (Table 1). There are two promising positive clones: CDC20 (cell cycle division 20 homolog) and NDRG1 (N-myc downstream regulated gene 1). CDC20 functions as a regulator of the M phase of the cell cycle, while NDRG1 plays a role in hypoxyia. We then performed the GeneGo program to analyze the network among the interacting proteins and Pir51 (figure 10).

Table 1 positive clones interact with Pir51

Screening	total
菌落篩選總數	57371 個
最初出現在 3-AT 篩選培養基菌落數	345
最終出現在 3AT 篩選培養基菌落數	147

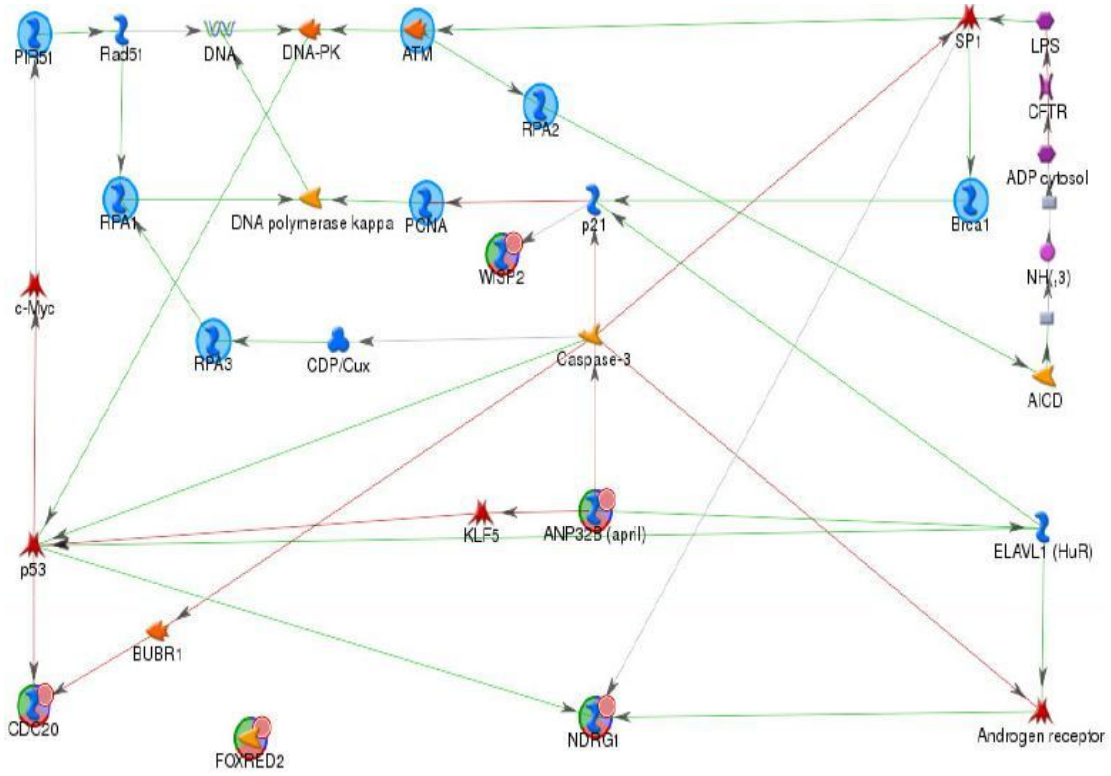


Figure 10. The pir51 protein complex network. Positive Pir51 associated proteins are analyzed via the GeneGo software program.

Characterizing the interacting proteins of the Pir51 protein:

We examined two positive clones: CDC20 and NDRG1 by using the immunostaining method. Indeed, we found pir51 co-localizes with CDC20 (figure 11) and NDRG1 (figure 12). In addition, Pir51 interacts with endogenous CDC20 (figure 13).

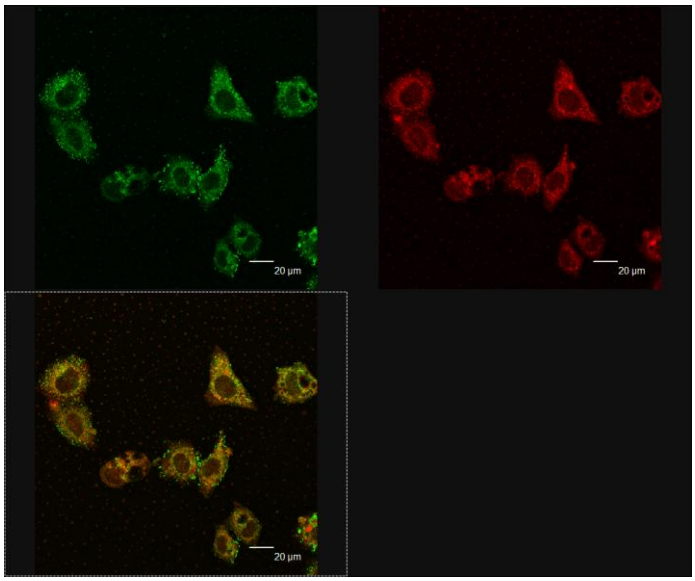


Figure 11. Pir51 co-localizes with CDC20. We performed the immunostaining assay and found Pir51 co-localizes with CDC20. Red: Pir51, Green: CDC20.

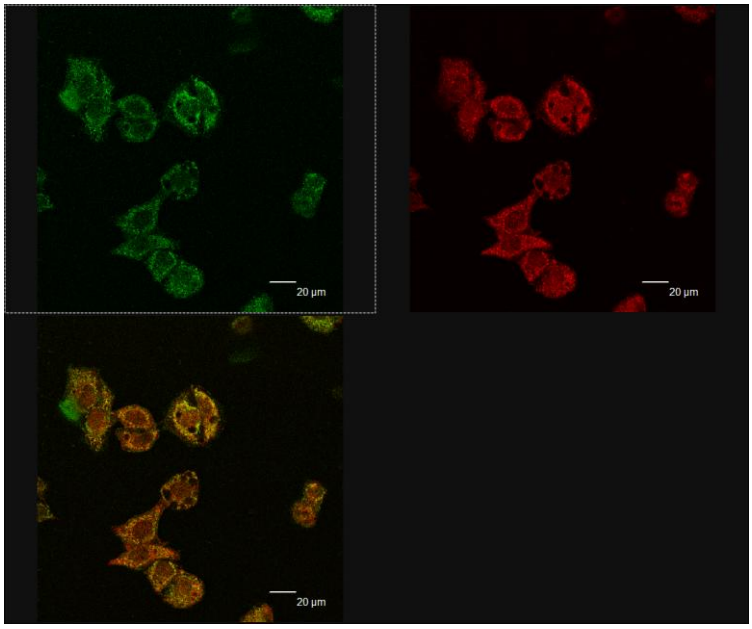


Figure 12. Pir51 co-localizes with NDRG1. We performed the immunostaining assay and found Pir51 co-localizes with NDRG1. Red: Pir51, Green: NDRG1.

Figure 13. Pir51 interacts with CDC20. We performed the co-IP assay and found CDC20 in the Pir51 protein complex. Lane 1: 2% HeLa lysate imput, lane 2: no antibody, lane 3: IP with anti-pir51 antibody.

CDC20 與 Pir51 有交互作用的區域(CDC20 interacting domain)

為了進一步知道 CDC20 與 Pir51 彼此交互作用的關係，我們從 CDC20 之 5’端開始，設計了長度 400 個鹼基對以及 500 個鹼基對的 DNA，以及 CDC20 的 WD-40 repeats 的部分，另外也設計一段 CDC20 的全長(1.5Kb)，總共四段，來探討 Pir51 與不同 region 之 CDC20 之間的功能性。由圖 14a 來看，是四條不同長度的 Flag-CDC20 的 total cell lysate，用來確定每段 Flag-CDC20 皆會表現，由左而右分別是 CDC20 的全長 1500bp、500bp、400bp、WD-40 repeats 共四段。由 Figure 14b 來看，可以得到一個結論，Pir51 只會與 CDC20 的全長有交互作用。

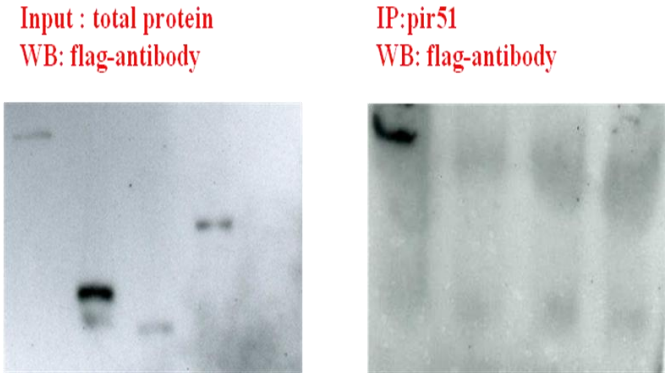
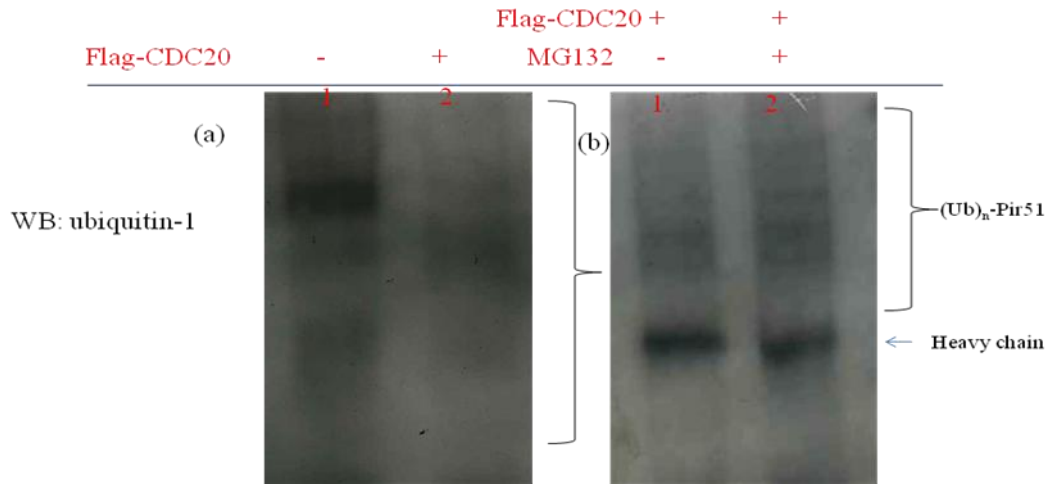
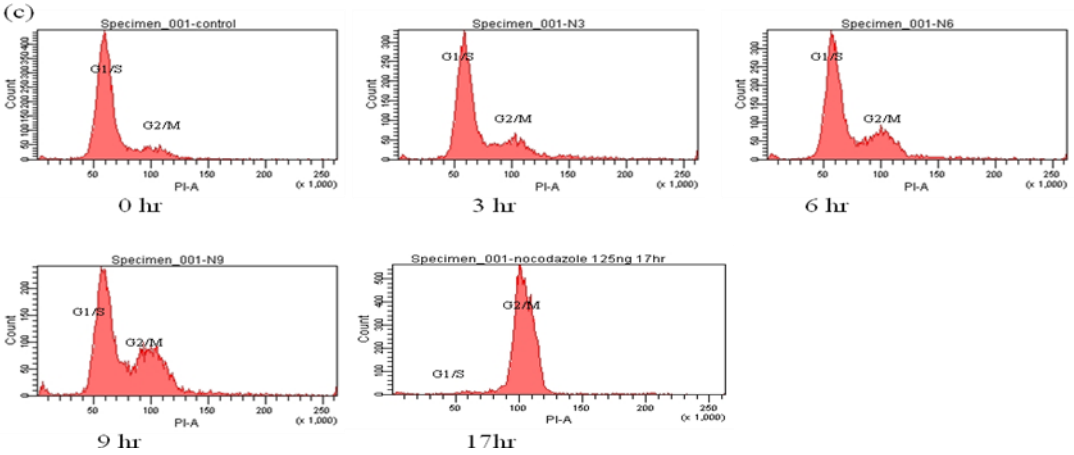


Figure 14 探討 CDC20 會與 Pir51 有交互作用的區域。(a)由左至右分別是四段不同長度 CDC20 的 total protein，分別是 CDC20 全長 1500bp、從 CDC20 的 N 端 500bp、CDC20 的 N 端 400bp、CDC20 的 WD-40 repeats 995bp，以證明 Flag-CDC20 有表現。(b) 由左至右分別是 (圖 14a)中的四個不同長度的 Flag-CDC20，IP 加入 Pir51，Western 以 Flag-antibody 來偵測，發現 Pir51 會與 Flag-CDC20 的全長有交互作用。

Pir51 會經由 APC^{CDC20} 而被泛蛋白化



(c)



(d)

- MVRPVRHKKPVNYSQFDHSDSDDDFVSATVPLNKKSRTAPKELKQDKPK PNLNNLRKEEIPVQEKTPKKRLPEGTFSIPASAVPCTKMALDDKLYQRDLE VALALSVKELPTVTTNVQNSQDKSIEKHGSSKIETMNKSPHISNCSVASDYL DLDKITVEDDVGGVQGKRKAASKAAAQQRKILLEGSDGDSANDTEPDFAP GEDSEDDSDFCESEDNDEDFSMRKSKVKEIKKKEVKVKSPVEKKEKKSKSKC NALVTSVDSAPAAVKSESQSLPKKVSLSSDTTRKPLEIRSPSAESKKPKWVPPAA SGGSRSSSSPLVVVSVKSPNQSLRLGLSRLARVKPLHPNATST

D(Destruction) box: RXXL
D box and KEN box are required for efficient ubiquitination by APC/C.

(e)

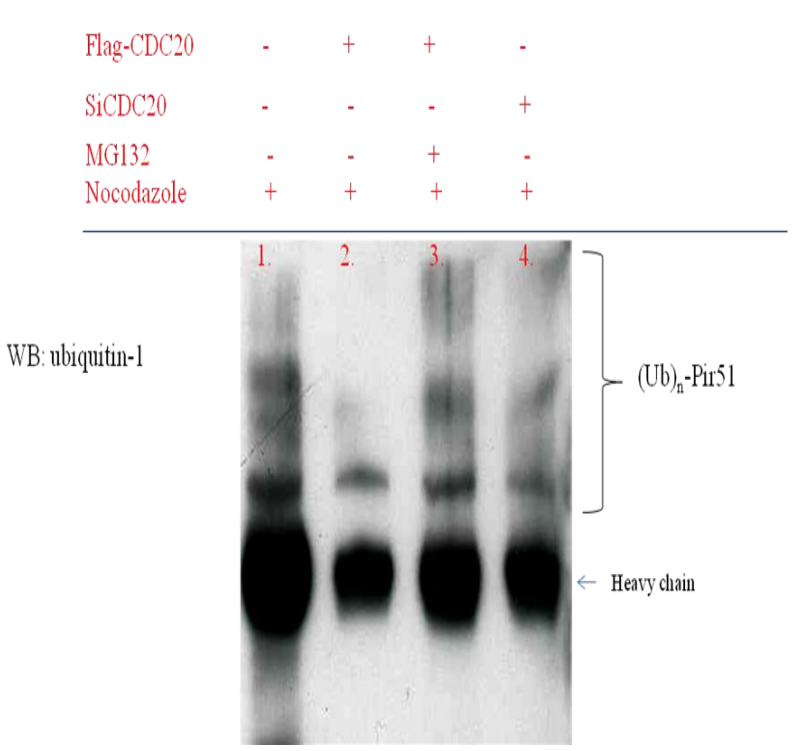


圖 15　Pir51 會經由 APC^{CDC20} 而被泛蛋白化。 (a)左邊是 HeLa 細胞，右邊是有轉染 Flag-CDC20 的細胞，以 ubi-1 抗體來觀察 Pir51 細胞被 ubiquitination 的情形。(b)左邊及右邊皆有轉染 Flag-CDC20 的細胞，右邊另外添加了 MG132，MG132 是 proteosome 的抑制劑。(c)以 nocodazole 處理細胞 17 小時後，可以發現細胞週期停在 M 期。(d) Pir51 的序列。(e)以 Ubi-1 抗體來觀察 Pir51 在不同條件下表現的情形，由左而右分別是:HeLa 細胞、轉染 Flag-CDC20 的細胞、轉染 Flag-CDC20 的細胞並且加入 MG132 處理、轉染 siCDC20 的細胞。

To determine the defects of Pir51 in the DNA damage response

◆ 人類全基因表現晶片

Symbol	Log值	倍數(sipir51/Hela)	Definition
IFI27	5.691895026	51.69292833	Homo sapiens interferon, alpha-inducible protein 27 (IFI27), transcript variant 2, mRNA.
IFI35	1.296153073	2.455731912	Homo sapiens interferon-induced protein 35 (IFI35), mRNA.
IFI44	2.2988058	4.920502991	Homo sapiens interferon-induced protein 44 (IFI44), mRNA.
IFI44L	3.615098232	12.25329827	Homo sapiens interferon-induced protein 44-like (IFI44L), mRNA.
IFI6	4.644364808	25.00881524	Homo sapiens interferon, alpha-inducible protein 6 (IFI6), transcript variant 2, mRNA.
IFI6	2.513261148	5.709091356	Homo sapiens interferon, alpha-inducible protein 6 (IFI6), transcript variant 3, mRNA.
IFIH1	2.038065739	4.10694532	Homo sapiens interferon induced with helicase C domain 1 (IFIH1), mRNA.
IFIT1	1.197754017	2.293822913	Homo sapiens interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), transcript variant 2, mRNA.
IFIT2	1.507935622	2.844027909	Homo sapiens interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), mRNA.
IFIT3	1.9663796	3.907862213	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
IFIT3	2.188388835	4.557961803	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
IFIT3	1.5045173	2.837297244	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
IFITM1	1.338021762	2.528044331	Homo sapiens interferon induced transmembrane protein 1 (9-27) (IFITM1), mRNA.
IFITM3	1.006413319	2.008910539	Homo sapiens interferon induced transmembrane protein 3 (1-8U) (IFITM3), mRNA.
IRF1	1.598669835	3.028639439	Homo sapiens interferon regulatory factor 1 (IRF1), mRNA.
IRF7	1.370884723	2.5862912	Homo sapiens interferon regulatory factor 7 (IRF7), transcript variant b, mRNA.
IRF7	2.265306548	4.807565616	Homo sapiens interferon regulatory factor 7 (IRF7), transcript variant b, mRNA.
IRF9	1.759111053	3.384894931	Homo sapiens interferon regulatory factor 9 (IRF9), mRNA.
CDKN1A	1.003625669	2.005032566	Homo sapiens cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), transcript variant 1, mRNA.

圖 16 人類全基因表現晶片分析結果-選出 Pir51 表現量下降量最高的細胞後，抽取 RNA，並交由均泰生技公司做人類全基因表現晶片。

此為**人類全基因表現晶片**的分析結果(部分)，我們選擇與細胞週期相關的基因 P21 (CDKN1A)，和干擾素相關基因，做進一步的研究(圖 17)。

◆ 利用 Metacore 軟體分析相關路徑圖

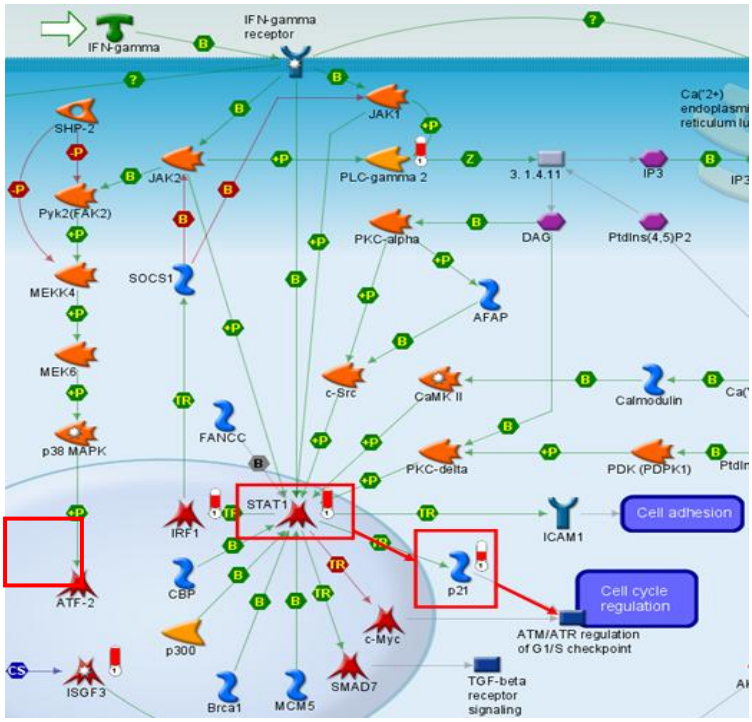


圖 17 Metacore 分析人類全基因表現晶片相關路徑結果-由此軟體分析晶片結果後，可以觀察到將 Pir51 knockdown 後主要影響的是與細胞週期調節相關的基因，於是選擇 P21(CDKN1A)，和干擾素相關的基因，做為主要的研究方向。

◆ 利用 Western blotting 觀察將 Pir51 knockdown 後相關基因的表現量

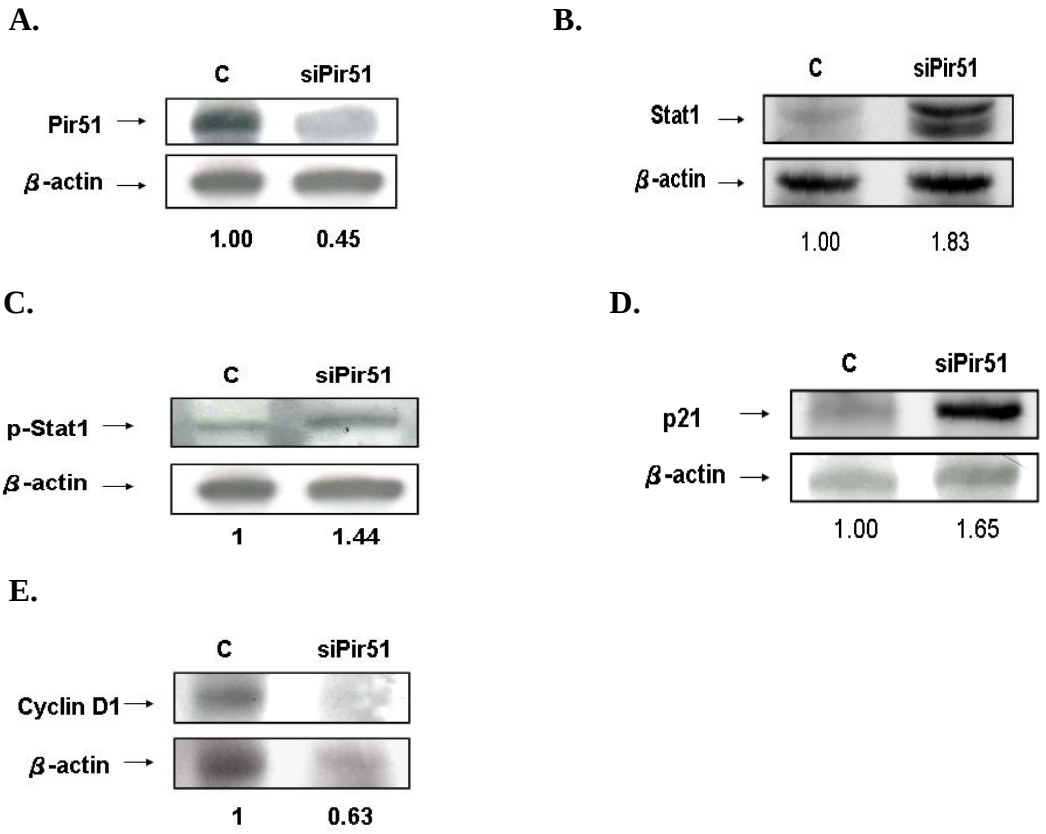
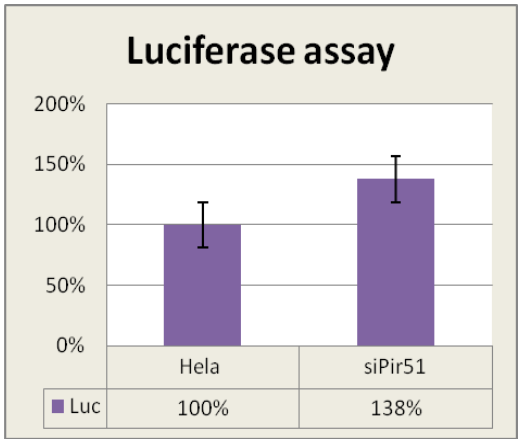


圖 18. Western blotting 結果 —利用 Western blotting 觀察將 Pir51 knockdown 後相關基因的蛋白質表現量。A:利用 shRNA 將 pir51 knockdown; B,C:將 Pir51 knockdown 後，Stat1 及 p-Stat1 表現量上升分別是 1.83 及 1.44 倍；D,E: 將 Pir51 knockdown 後，P21 上升約 1.65 倍,而 CyclinD1 表現量下降了百分之三十七。



**p-value 0.02067*

圖 19. Dual-luciferase reporter assay 結果。 由此圖可看見將 Pir51 knockdown 後，ISRE 之活性上升百分之三十八(**p-value 0.02067*)。

◆ 流式細胞儀觀察 knockdown pir51 時細胞週期的改變

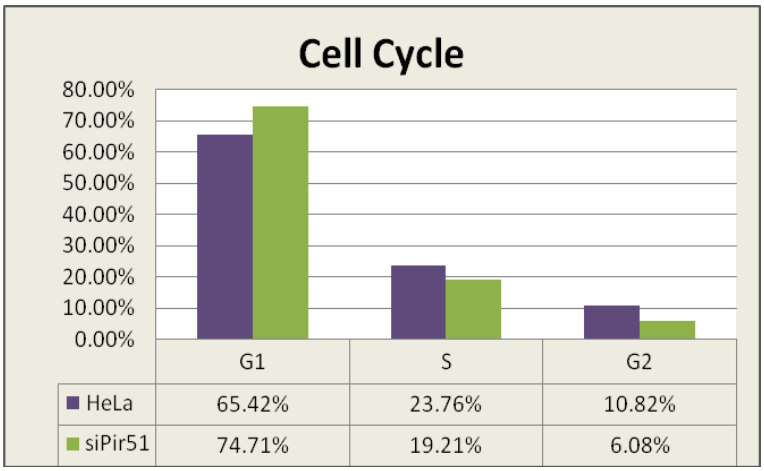


圖 20 流式細胞儀觀察細胞週期結果。圖中顯示當 Pir51 表現量下降後，G1 期比例上升從 65.4% 上升至 74.71%。

由於以上各結果顯示將 Pir51 knockdown 後，皆可觀察到 P21 此細胞週期相關基因蛋白質及 mRNA 表現量皆會上升，並且 Western blotting 結果顯示其下游 CyclinD1 蛋白質表現量下降，過去文獻指出 p21 的活化能進而導致細胞週期停滯於 G1 期。因此我們利用流式細胞儀，觀察將 Pir51 knockdown 後對影響細胞週期的影響，結果顯示當 Pir51 表現量下降後，G1 期比例上升從 65.4%上升至 74.71% (圖 20.)。於是我們可以推測，Pir51 表現量下降，可能會經由活化 P21 此條途徑，進而使細胞週期停滯於 G1 期。

結論

經由多種實驗方法可以發現，knockdown Pir51 可使細胞中的 Stat1,P21 相關基因表現量上升，並且觀察到其下游 CyclinD1,Ifi27 皆會受影響，因此可推知 Pir51 與細胞週期的表現有關聯性。我們可以初步推測 Pir51 在細胞內所參與的途徑之一，可能是藉由活化 Stat1，進而調控 P21 的轉錄，並經由 CyclinD1 影響細胞週期停滯於 G1 期。至於 Pir51 是如何影響 Stat1，此分子機制尚不清楚，這也是我們有興趣想探討的一部分，接著我們也會探討此些相關基因在不同癌症細胞之間的表現情況，進而探討 Pir51 與癌症之間的關聯性。

2. 參考文獻

1. Kovalenko OV, Golub EI, Bray-Ward P, Ward DC, Radding CM. (1997). A novel nucleic acid-binding protein that interacts with human rad51 recombinase. Nucleic Acids Res. 15;25(24):4946-53.

2. Henson, S.E., Tsai, S.C., Malone, C.S., Soghomonian, S.V., Ouyang, Y., Wall, R., Marahrens, Y., and Teitell, M.A. (2006). Pir51, a Rad51-interacting protein with high expression in aggressive lymphoma, controls mitomycin C sensitivity and prevents chromosomal breaks. Mutation research 601, 113-124.

3. Wiese C, Dray E, Groesser T, San Filippo J, Shi I, Collins DW, Tsai MS, Williams GJ, Rydberg B, Sung P, Schild D. (2007). Promotion of homologous recombination and genomic stability by RAD51AP1 via RAD51 recombinase enhancement. Mol Cell. 28(3):482-90.

4. Modesti M, Budzowska M, Baldeyron C, Demmers JA, Ghirlando R, Kanaar R. (2007). RAD51AP1 is a structure-specific DNA binding protein that stimulates joint molecule formation during RAD51-mediated homologous recombination. Mol Cell. 28(3):468-81.

5. Obama K, Satoh S, Hamamoto R, Sakai Y, Nakamura Y, Furukawa Y. (2008)歐 Enhanced expression of RAD51 associating protein-1 is involved in the growth of intrahepatic cholangiocarcinoma cells. Clin Cancer Res. 2008 Mar 1;14(5):1333-9.

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□ 未達成目標（請說明，以 100 字為限）

□ 實驗失敗

□ 因故實驗中斷

□ 其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

我們的研究使用蛋白質二維電泳-質譜儀，免疫共沉澱，和免疫染色法發現 pir51 與許多 DNA 修補蛋白結合(例如: ATM, ATM,and Rad51)，並且與 DNA 複製蛋白(例如: RPA32, and RPA70)有交互作用。我們的使用細菌雙雜交法，發現 pir51 與 CDC20 有交互作用。進一步發現 pir51 會被泛素化(ubiqitination)是經由 CDC20 蛋白複合體。此外，在無 pir51 表現時，STAT1 (Signal Transducers and Activators of Transcription) 和 p21 表現量增加。STAT1 屬於 STAT 家族中的一員，這類家族蛋白調控上游細胞激素 (cytokine)上游細胞激素的訊息傳遞，進而調節下游對應基因的表現。p21 是 p53(腫瘤抑制基因)的下游基因，參與細胞週期的調控。我們的發現提供一個新的方向在 DNA 修補、細胞週期、和基因調控。我們的發現期許提供一個新的視野在腫瘤治療及偵測。