

**Inhibitors identify an auxiliary role for mTOR signalling in necroptosis execution downstream of MLKL activation**

Sarah E. Garnish<sup>1,2,\*†</sup>, Christopher R. Horne<sup>1,2,3</sup>, Yanxiang Meng<sup>1,2</sup>, Samuel N. Young<sup>1</sup>, Annette V. Jacobsen<sup>1,2</sup>, Joanne M. Hildebrand<sup>1,2</sup> and James M. Murphy<sup>1,2,3\*</sup>

<sup>1</sup> Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia

<sup>2</sup> Department of Medical Biology, University of Melbourne, Parkville, VIC 3052, Australia

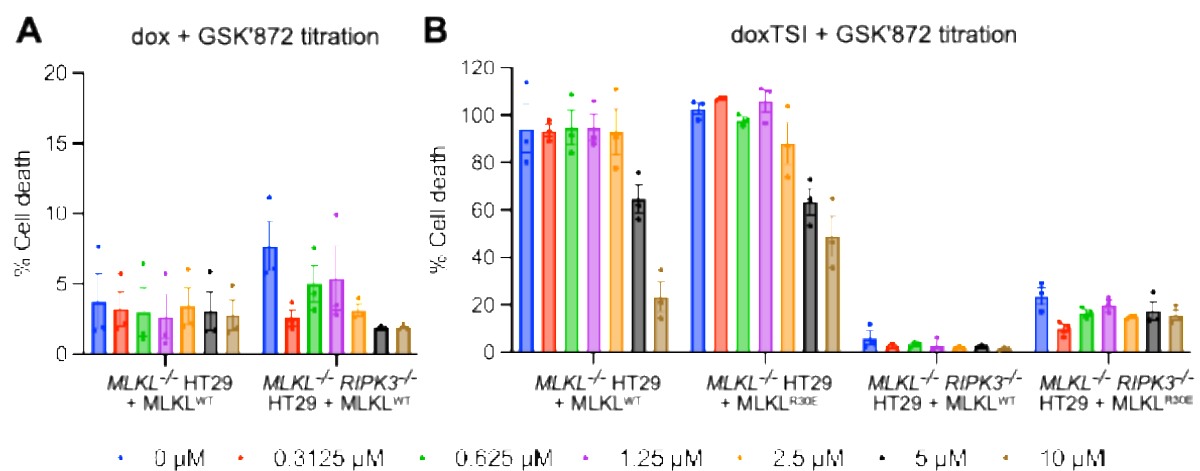
<sup>3</sup> Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia

\* To whom correspondence may be addressed [sarah.garnish@monash.edu](mailto:sarah.garnish@monash.edu) and [jamesm@wehi.edu.au](mailto:jamesm@wehi.edu.au)

† Current address: Monash Biomedicine Discovery Institute, Department of Microbiology, Monash University, Clayton, Victoria, 3800

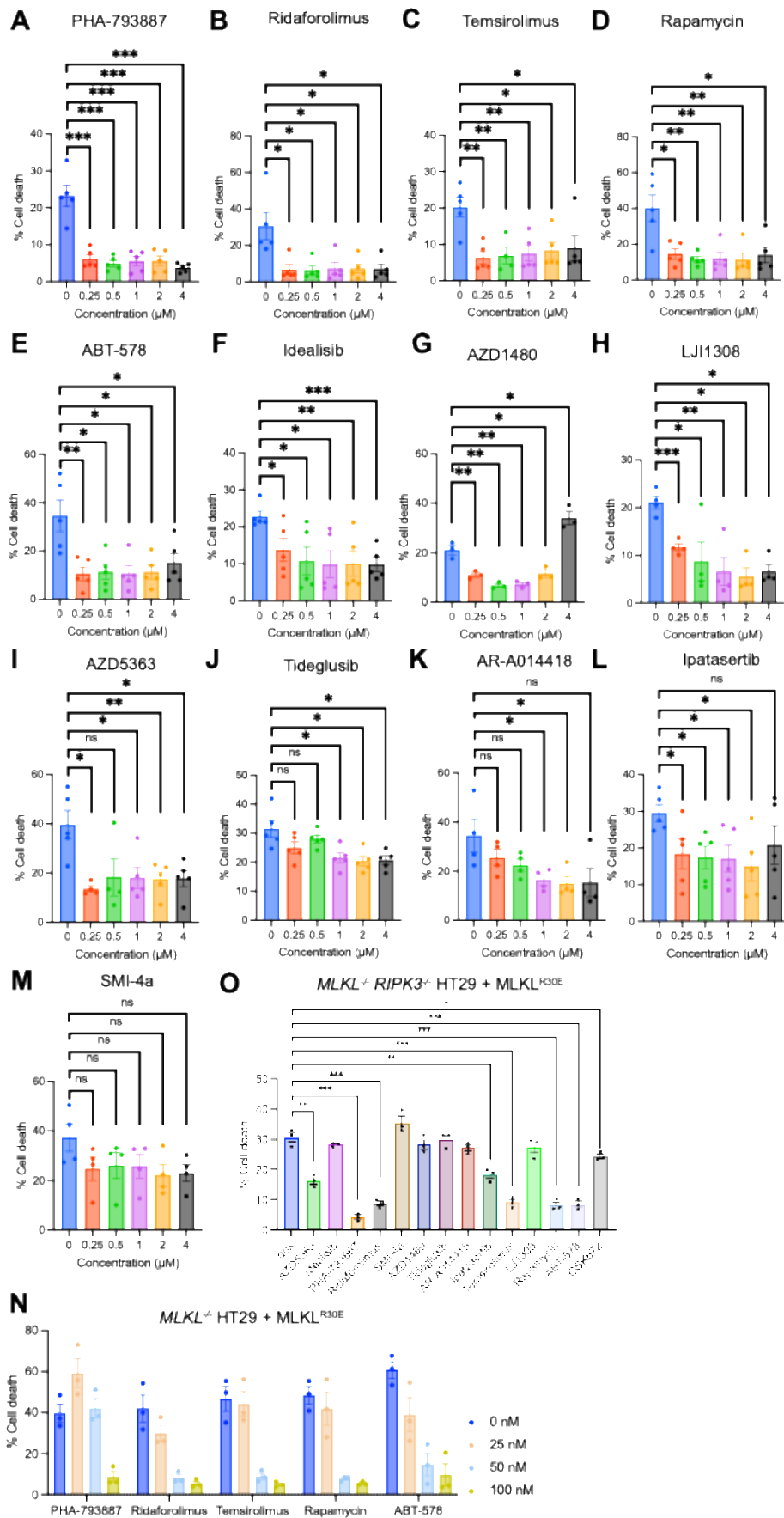
**Keywords:** RIPK1, RIPK3, mTORC1, MLKL, cell death

**Supplementary Figure 1. GSK'872 inhibits MLKL WT executed necroptosis at high concentrations.** **A - B** *MLKL*<sup>-/-</sup> and *MLKL*<sup>-/-</sup> *RIPK3*<sup>-/-</sup> HT29 cells stably transduced with *MLKL*<sup>WT</sup> or *MLKL*<sup>R30E</sup> were treated with doxycycline (Dox) alone or in combination with TSI (TNF, Smac mimetic, IDN-6556), in the presence of RIPK3 kinase inhibitor, GSK'872, at increasing concentrations. IncuCyte SX5 imaging was used to quantify the percentage of cell death by determining the number SYTOX Green-positive cells (dead cells) relative to the number of DRAQ5-positive cells (total cell number) at 48 h. Data are plotted as mean ± SEM of *n* = 3. Two independent cell lines transduced with *MLKL*<sup>R30E</sup> were generated; one was assayed in *n* = 1 and the other in *n* = 2, for a total of *n* = 3, independent experiments.



**Supplementary Figure 2. Positive compound hits exhibit inhibition at different concentrations.**

**A – N** Evaluation of R30E MLKL constitutive death inhibition at varying compound concentrations. **(A – M)** *MLKL*<sup>-/-</sup> HT29 cells were stimulated with 100 ng/mL doxycycline to induce expression of MLKL R30E and stimulated concurrently with compound at 0.25 – 4  $\mu$ M. **(N)** *MLKL*<sup>-/-</sup> HT29 cells were co-stimulated with 100 ng/mL doxycycline and PHA-793887, Ridaforolimus, Temsirolimus, Rapamycin, or ABT-578 at 25, 50 or 100 nM. **(O)** *MLKL*<sup>-/-</sup>, *RIPK3*<sup>-/-</sup> HT29 cells were stimulated simultaneously with 1  $\mu$ M of compound and 100 ng/mL doxycycline to induce MLKL R30E expression. **(A – O)** Cell death was quantified at 44 hours as percentage by determining the number SYTOX Green-positive cells (dead cells) relative to the number of DRAQ5-positive cells (total cell number) using IncuCyte SX5 live cell imaging. Cells were assayed in  $n = 3$  (**G, N, O**),  $n = 4$  (**H, I, M**),  $n = 5$  (**A, B, C, D, F, K, L**) or  $n = 6$  (**E, J**) independent experiments with data plotted as mean  $\pm$  SEM.  $P$  value calculated using an unpaired, two-tailed Students  $t$ -test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$



**Supplementary Table 1. 429 screened kinase inhibitors**

| <b>Compound</b>                 | <b>Fold decrease in % cell death</b> |
|---------------------------------|--------------------------------------|
| Chloroquine phosphate           | -0.0405249                           |
| GSK621                          | -0.0060663                           |
| xMD8-92                         | 0.24858143                           |
| GSK2636771                      | 0.06102663                           |
| AMG337                          | -0.4311096                           |
| ETC-1002                        | 0.04173598                           |
| PRT062607 (P505-15 BllB057) Hcl | 0.07542861                           |
| GSK2292767                      | 0.21820026                           |
| LY2228820                       | -0.0468782                           |
| 7 8-dihydroxyflavone            | -0.3046597                           |
| GSC-0349                        | 0.40044252                           |
| Tyrphostin AG 1296              | -0.0939585                           |
| PQ 401                          | -0.4659934                           |
| Cobimetinib                     | -1.0609905                           |
| Butein                          | 0.0553905                            |
| Olmutinib                       | -0.2244427                           |
| SMI-4a                          | 0.24885357                           |
| AZD3759                         | -0.2009471                           |
| ASP3026                         | -0.0039963                           |
| PLX7904                         | -0.0106042                           |
| Entrectinib                     | -0.38863                             |
| CC-223                          | 0.60982089                           |
| TCS 359                         | 0.27914002                           |
| Mitefosine                      | -0.204019                            |
| Pacritinib                      | -1.9440588                           |
| Palbociclib                     | -0.120877                            |
| LY3023414                       | -0.1827803                           |
| VPS34-IN1                       | -0.462318                            |
| VE-821                          | -0.622079                            |
| AZD5363                         | 0.54575239                           |
| APTSTAT3-9R                     | -0.3840816                           |
| Oclacitinib                     | 0.21073002                           |
| P276-00                         | 0.13713979                           |
| Prerifosine                     | -0.1192926                           |
| ETP-46464                       | -0.3244138                           |
| GSK2292767                      | 0.14275473                           |
| Dorsomorphin 2HCl               | -1.4559273                           |
| Oimutinib                       | -0.091818                            |
| Mitefosine                      | -0.0398052                           |
| ONO-4059                        | -0.0139509                           |
| AZD2858                         | -0.9997674                           |

|                  |            |
|------------------|------------|
| PP1              | 0.13339578 |
| GNE-9605         | 0.02412315 |
| SGI-7079         | -0.8281839 |
| LFM-A13          | -0.0457122 |
| JNK inhibitor IX | 0.16872134 |
| LDN-214117       | -0.2912278 |
| MLN2480          | -0.5729607 |
| SSR128129E       | -0.0910189 |
| PD173955         | -0.811333  |
| GNF-5            | -0.2567442 |
| CC-292           | -0.708888  |
| RN486            | -0.0433536 |
| Certinib         | -0.5518132 |
| Osimertinib      | -0.1975361 |
| FIIN-2           | 0.22636921 |
| CGI1746          | -0.6019664 |
| PF-431396        | -0.0176005 |
| G-749            | -0.2868299 |
| DDR1-IN-1        | -0.052458  |
| ANA-12           | -0.0723448 |
| CNX-774          | 0.07325858 |
| CNX-2006         | -0.0569623 |
| Rociletinib      | -0.1205487 |
| WH-4-023         | -0.0774249 |
| AZD3463          | 0.0773221  |
| PF-3758309       | -0.2568879 |
| abemaciclib      | -0.2032947 |
| SU9516           | 0.05573734 |
| Akt1-1/2         | -0.1004359 |
| D4476            | -0.0073294 |
| VE-822           | -0.0797677 |
| URMC-099         | -0.7460842 |
| LJI308           | 0.25707804 |
| Anacardic Acid   | 0.20765506 |
| KN-62            | 0.01000502 |
| IPA-3            | 0.05931088 |
| MK-8745          | -0.0205436 |
| Purvalanol A     | 0.15712694 |
| LY2090314        | -0.188939  |
| AMG319           | 0.02722871 |
| AZD6738          | 0.2477438  |
| BLZ945           | -0.0144258 |
| SC1              | -0.5212712 |

|                       |            |
|-----------------------|------------|
| 10058-F4              | 0.08855985 |
| TF009                 | 0.02680453 |
| IM-12                 | 0.06143401 |
| LJH685                | 0.08565436 |
| Bikinin               | 0.03050886 |
| Ro-3306               | 0.08129365 |
| Ulixertinib           | -0.390547  |
| SU6656                | -0.8620859 |
| TIC10                 | 0.01842245 |
| AZD1080               | -0.0119338 |
| AT13148               | 0.13175824 |
| XMD8-92               | -0.232065  |
| Ro3280                | -0.8234966 |
| SKI II                | -0.1893463 |
| Losmapimod            | -0.0505795 |
| GNE-0877              | -0.6233195 |
| Sorafenib             | -0.2853192 |
| HS-173                | -0.5947267 |
| CGK 733               | 0.21057786 |
| KN-93 phosphate       | -0.1918577 |
| Ehop-016              | -0.6734572 |
| FRAX597               | -0.482735  |
| Urprosertib           | 0.1849065  |
| Skepinone-L           | 0.10665542 |
| PF 543                | -0.0933528 |
| WZ4003                | -0.6960127 |
| Bay 11-7085           | 0.20520488 |
| GSK2334470            | 0.0068028  |
| ERK5-IN-1             | -0.7624297 |
| Tepotinib             | 0.10167927 |
| CX-6258 Hcl           | -0.1801118 |
| SB239063              | 0.16655392 |
| AZD1208               | 0.16216467 |
| Zotarolimus (ABT-578) | 0.32053149 |
| HTH-01-015            | -0.2235406 |
| RKI-1447              | 0.04201273 |
| AZ20                  | 0.30536356 |
| Bisindoylmaleimide I  | -0.7560681 |
| Fingolimod HCL        | -0.3911107 |
| ZM 306416             | 0.02011049 |
| AZD4547               | -1.3672333 |
| PD168393              | -0.0388351 |
| Golvatinib            | -0.0749811 |

|                     |            |
|---------------------|------------|
| Crenolanib          | -0.1666422 |
| PF-04691502         | -0.0407915 |
| NVP-BVU972          | 0.15643816 |
| ZM 336372           | 0.10239138 |
| VS-5584             | -0.3366192 |
| GSK1838705A         | 0.12484658 |
| Trametinib          | -0.4750527 |
| TAK-285             | 0.25125281 |
| PP2                 | 0.14244941 |
| AG-1478             | 0.00033954 |
| Ibrutinib           | -0.1595404 |
| Facomitinib         | -0.4543782 |
| ALK-IN-1            | -0.0394632 |
| GNF-2               | -0.0823925 |
| Dabrafenib          | -0.5367798 |
| KX2-391             | -0.3868299 |
| ZM 323881           | 0.02420811 |
| AS-604850           | 0.11326876 |
| Milciclib           | -0.4253718 |
| Binimetinib         | -0.2592474 |
| JNK-IN-8            | -0.0921086 |
| TAE226              | 0.07951178 |
| AMG-458             | -0.0377978 |
| Torin 2             | 0.12190223 |
| PF-00562271         | 0.16561367 |
| Dovitinib           | -0.2326487 |
| CHIR-98014          | 0.10220641 |
| Cabozantinib malate | 0.15151577 |
| TPCA-1              | -0.236893  |
| SC-514              | -0.089076  |
| Semaxanib           | 0.19168874 |
| TAK-715             | 0.22346325 |
| BI-D1870            | 0.03586908 |
| PF-477736           | -0.7352786 |
| Varlitinib          | 0.43822617 |
| Icotinib            | 0.07387357 |
| WAY-600             | -0.1781234 |
| Tideglusib          | 0.26792124 |
| IMD0354             | -0.5333861 |
| CEP-33779           | 0.03802728 |
| PH-797804           | 0.15391477 |
| VX-702              | 0.03903298 |
| Dinaciclib          | -0.0182165 |

|                   |            |
|-------------------|------------|
| S-Ruxolitinib     | -0.0184934 |
| SB415286          | 0.09443759 |
| Flavopiridol Hcl  | 0.01913586 |
| 3-Methyladenine   | 0.10578959 |
| A-769662          | 0.02037678 |
| PHA-767491        | 0.24659652 |
| Tyrphostin 9      | -0.6758379 |
| Ipatasertib       | 0.23257124 |
| WP1066            | 0.31459776 |
| R547              | 0.18082941 |
| Duvelisib         | 0.48512327 |
| Vistusertib       | 0.0344859  |
| Tofacitinib       | -0.0711437 |
| GW5074            | -0.3445876 |
| TAK-901           | -0.481742  |
| Tofacitinib       | -0.1123875 |
| MK-5108           | -0.0399101 |
| Sotrastaurin      | -0.8015436 |
| CAY10505          | -1.0901069 |
| Fedratinib        | -1.2711399 |
| AMG-900           | -2.4212745 |
| PF-562281         | 0.26083329 |
| Apitolisib        | -0.3998758 |
| CH5132799         | -0.1420317 |
| Go 6983           | -0.6138957 |
| WHI-P154          | -0.0361985 |
| SAR131675         | -0.0420243 |
| TF101209          | -0.5352343 |
| Tyrphostin AF 879 | -1.8978113 |
| CHIR-124          | -1.4574208 |
| CUDC-907          | -1.5462262 |
| Alpelisib         | -0.0820406 |
| INK 128           | -0.3479076 |
| TDZD-8            | -0.3917955 |
| CHIR-99021        | -0.1930263 |
| AZ628             | -0.3723608 |
| XL019             | -0.0611435 |
| Tivantinib        | -0.1671339 |
| BAY 11-7082       | 0.08907177 |
| MK-2461           | -0.8053556 |
| NVP-BSK805 2HCL   | -0.7576843 |
| Pazopanib         | -0.008006  |
| Schisandrin B     | 0.13268614 |

|                        |            |
|------------------------|------------|
| CZC24832               | -0.0492883 |
| IKK-16                 | -0.7828093 |
| Fasudil                | -0.2683456 |
| KU-60019               | -0.4228989 |
| AST-1306               | -1.0498926 |
| BMS-777607             | -0.0710016 |
| Pimasertib             | -0.6412906 |
| AT7867                 | -0.5093821 |
| AZD8330                | -1.2675819 |
| R406                   | -0.3525537 |
| KRN 633                | 0.04667716 |
| CCT128930              | 0.21839026 |
| Volasertib             | -0.7851232 |
| Doramapimod (BIRB 7960 | 0.33644795 |
| SP600125               | -0.4381002 |
| H89 2HCL               | -0.0908502 |
| CP-673451              | -0.1274372 |
| AICAR                  | 0.08580934 |
| PD318088               | -0.1414709 |
| Ki8751                 | 0.21710291 |
| PF-573228              | 0.00478819 |
| Quizartinib            | -0.288081  |
| Apatinib               | -0.1004807 |
| Genistein              | -0.0572942 |
| PF-4708671             | 0.2285693  |
| AT7519                 | -0.4548543 |
| Lapatinib              | -0.3038144 |
| OSI-420                | 0.04933999 |
| R406                   | 0.08560969 |
| AS-252424              | -0.369015  |
| AZ960                  | -0.3554994 |
| Asiatic Acid           | -0.0288685 |
| Thiazovivin            | 0.23971213 |
| Hesperadin             | -2.5470527 |
| AZD8055                | -0.3219834 |
| Omipalisib             | 0.04308302 |
| AZD5438                | 0.17957679 |
| PHT-427                | 0.29123411 |
| BMD-265246             | 0.20461036 |
| Degrasyn               | 0.45064509 |
| BS-181 Hcl             | -0.2370265 |
| A-674563               | 0.16655665 |
| Tie2 kinase inhibitor  | 0.42396368 |

|                      |            |
|----------------------|------------|
| BIX 02189            | 0.03677388 |
| Voxtalib             | -0.0366953 |
| PHA-793887           | 0.28965955 |
| KW-2449              | -0.3265447 |
| Phenformin HCl       | -0.1711074 |
| PIK-294              | -0.1079404 |
| PIK-93               | -0.515252  |
| SB590885             | -0.0816097 |
| Gandotinib           | -0.6923412 |
| LY2603618            | -0.1519462 |
| OSI-027              | 0.13370723 |
| HMN-214              | 0.08893952 |
| GSK461364            | -0.9226705 |
| BIX 02188            | -0.0469689 |
| PIK-93               | -0.3562587 |
| SB590885             | -0.2408273 |
| Gandotinib           | 0.01085854 |
| AZD1480              | 0.25964876 |
| Idealisib            | 0.27723881 |
| GSK492286A           | 0.04071098 |
| Torkinib             | -0.3598426 |
| AEE788               | -0.0625116 |
| Pelitinib            | -0.3895753 |
| PIK-293              | 0.4117966  |
| Quercetin            | 0.08650997 |
| TWS119               | 0.00559156 |
| TAK-733              | -0.2889716 |
| Momelotinib          | -0.008504  |
| PHA-680632           | -2.2366734 |
| Daphnetin            | 0.14404944 |
| Rigosertib           | -0.4939113 |
| AZD6482              | 0.37713686 |
| SGI-1776             | -0.3441689 |
| Aurora A inhibitor I | -0.3417894 |
| AZD7762              | -0.2485455 |
| Indirubin            | -0.0453315 |
| Buparlisib           | 0.10350126 |
| PD0325901            | -0.3120149 |
| PF-04217903          | 0.02789165 |
| Dasatinib            | -0.9659683 |
| PD98059              | 0.04810609 |
| CP-724714            | -0.1736308 |
| Afatinib             | 0.05856454 |

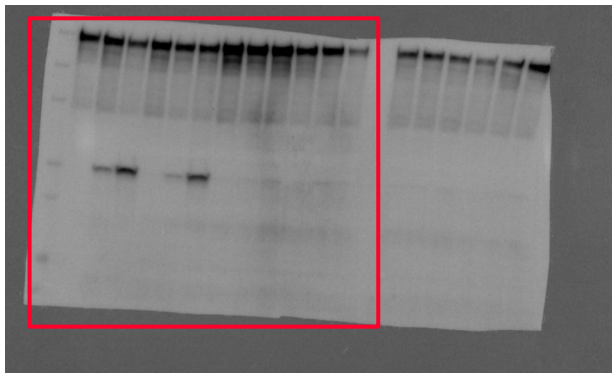
|                    |            |
|--------------------|------------|
| CYC116             | -0.1344925 |
| YM201636           | -0.3668497 |
| AG-490             | -0.0148836 |
| Refametinib        | -0.2226589 |
| Vemurafenib        | -0.6079179 |
| GSK690693          | 0.09069869 |
| BMS-536924         | -0.8046526 |
| Sunitinib Malate   | -1.4139638 |
| SNS-314 Mesylate   | -0.3608054 |
| Orantinib          | 0.02658796 |
| Honokiol           | 0.02460899 |
| MGCD-265           | -0.8667051 |
| WYE-125132         | -0.5607472 |
| RAF265             | -0.5281368 |
| Ponatinib          | -0.8761699 |
| BX-912             | -0.3461851 |
| A66                | -0.0650637 |
| Mubritinib         | 0.20577684 |
| PP121              | -0.3967916 |
| BMS-794833         | -0.0643706 |
| Fostamatinib       | -0.1608227 |
| NVP-BHG712         | -0.4974435 |
| Telatinib          | 0.02550119 |
| Chrysophanic Acid  | -0.1330479 |
| Rebastinib         | -0.0588676 |
| TGX-221            | 0.12422004 |
| Bosutinib          | -0.0204938 |
| ENMD-2076          | -1.5380366 |
| MLN8054            | -0.0249565 |
| BI2536             | -0.6663597 |
| Roscovitine        | -0.0033107 |
| CUDC-101           | -0.6027135 |
| CHIR-99021         | -0.421929  |
| PHA-665752         | -0.4489671 |
| JNJ-7706621        | -0.042166  |
| Barasertib         | -0.1408329 |
| BMS-754807         | -0.3183567 |
| Alisertib          | -0.1103674 |
| Brivanib Alaninate | -0.1882721 |
| Flavopiridol       | -0.3653941 |
| KU-0063794         | -0.1859941 |
| Everolimus         | 0.25145067 |
| PLX-4720           | 0.0823651  |

|                                     |            |
|-------------------------------------|------------|
| AG-1024                             | 0.01344679 |
| SB203580                            | -0.1631383 |
| Triciribine                         | 0.09456852 |
| SNS-032                             | -0.5186033 |
| WYE0354                             | 0.23316279 |
| LY294002                            | 0.27415703 |
| Y-27632                             | -0.0678873 |
| Temsirolimus                        | 0.22855436 |
| BMS-540215                          | 0.0567024  |
| BMS-599626                          | -0.0922905 |
| GDC-0941                            | -0.5156996 |
| SL-327                              | -0.0996373 |
| SB202190                            | 0.00237347 |
| PHA-739358                          | -0.7394955 |
| SU11274                             | -0.6806088 |
| OSU-03012                           | -0.7418398 |
| SB216763                            | -0.4450388 |
| Enzastaurin                         | -0.9312847 |
| MK-2206                             | 0.29925848 |
| Crizotinib                          | 0.01539736 |
| ZM 447439                           | -0.3267052 |
| U0126-EtOH                          | -0.1124054 |
| AZD2171                             | -0.3275906 |
| BIBF 1120                           | 0.0684369  |
| GDC-0879                            | -0.105675  |
| Rapamycin                           | 0.44045878 |
| Ridaforolimus (Deforolimus_MK-8669) | 0.43805374 |
| Linisitinib                         | -0.0608465 |
| ZSTK474                             | 0.35841469 |
| Tozasertib                          | -1.0658166 |
| Nilotinib                           | -0.2263975 |
| BX-795                              | -1.8839578 |
| OSI-930                             | 0.05806826 |
| Lenvantinib                         | 0.08492532 |
| PD173074                            | -0.096109  |
| BMS-907351                          | 0.03320073 |
| Dovitinib                           | -0.7440486 |
| Axitinib                            | -1.7599929 |
| PD184352                            | -0.1498909 |
| Tivozanib                           | 0.09892976 |
| WZ8040                              | 0.07589672 |
| Gefitinib                           | 0.07844606 |

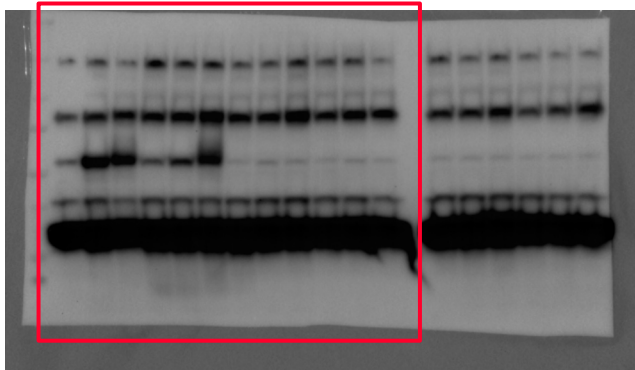
|                    |            |
|--------------------|------------|
| GSK1904529A        | -0.2467547 |
| NVP-AEW541         | 0.06956223 |
| BAY 7304506        | 0.11147687 |
| KU-55933           | -0.4206395 |
| Linifanib          | 0.00933075 |
| WZ4002             | -0.3572975 |
| JNJ-38877605       | -0.30357   |
| AMG-706            | -0.2456215 |
| GSK1363089         | -1.0023105 |
| AZD0530            | -0.8367411 |
| AB1010             | -0.3132263 |
| PI-103             | 0.07329861 |
| GW786034 HCL       | 0.21363329 |
| Vatalanib          | -0.0199897 |
| MP-470             | -0.363878  |
| AZD6244            | -0.2827635 |
| Sorafenib Tosylate | -0.0329636 |
| WZ3146             | -0.4650804 |
| Imatinib Mesylate  | 0.14064144 |
| GNE-7915           | -0.2342394 |
| NU6027             | 0.13216533 |
| GSK650394          | -2.0849086 |
| Vacquinol-1        | 0.06281515 |
| Piceatannol        | 0.00267276 |
| AZD2932            | -0.0356779 |
| GW441756           | -0.0022164 |
| AZD8931            | 0.19159713 |
| TG100-115          | 0.05037356 |
| AT9283             | -1.3717952 |
| GW-572016          | -0.0261566 |
| CEP-32496          | -0.0545657 |
| ZM 39923           | -0.0977243 |
| NSC 23766          | 0.06176297 |
| BMS-345541         | 0.14448733 |
| DASA-58            | -0.5943354 |
| AG-18              | -0.1193608 |
| BI-78D3            | -0.3188474 |
| Dorsomorphin 2HCl  | -1.5524144 |
| LDC000067          | 0.13271111 |
| VX-509             | 0.19074856 |
| GLPG0634           | -0.0526067 |
| TAK-632            | -0.0484299 |
| GZD824             | -0.5746653 |

|            |            |
|------------|------------|
| AR-A011418 | 0.26068044 |
| VX-11e     | -0.5339115 |

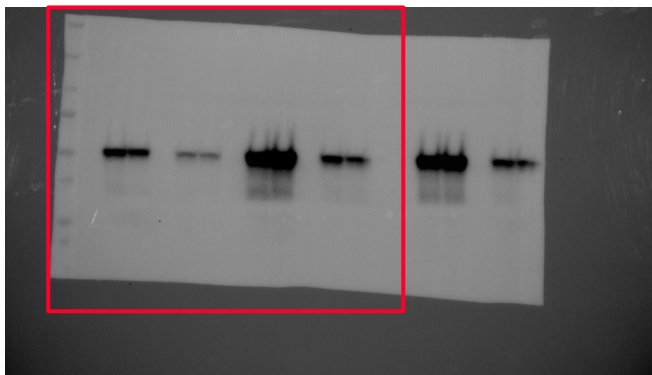
FIGURE 1C



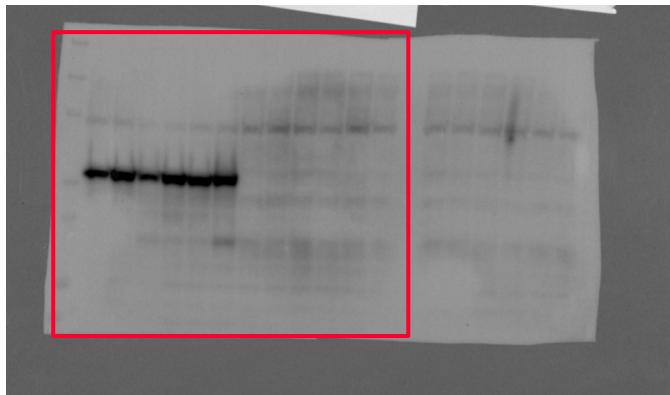
α-p.MLKL



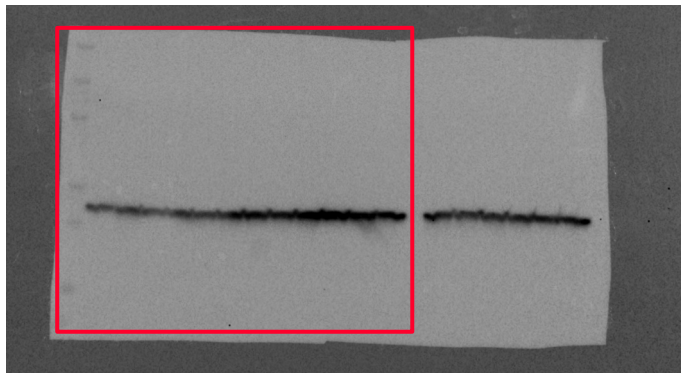
α-p.RIPK3



α-MLKL

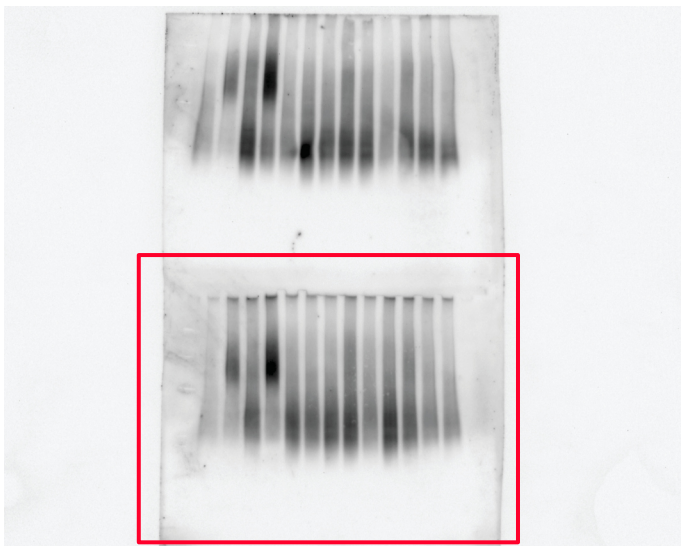


α-RIPK3

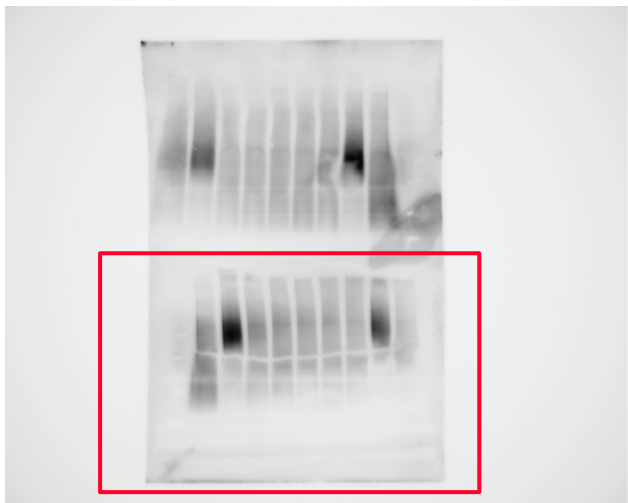


α-Actin

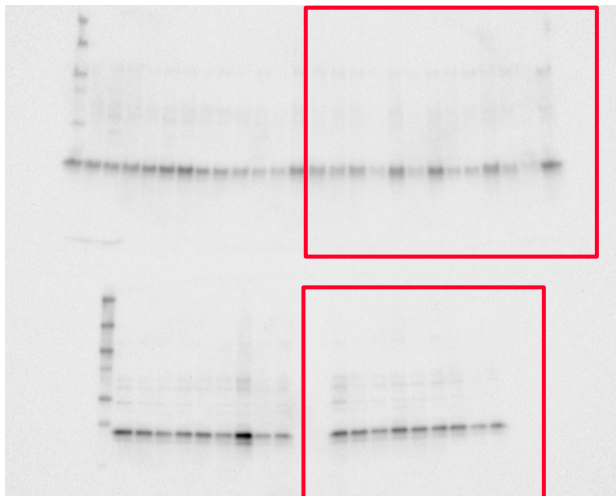
FIGURE 5



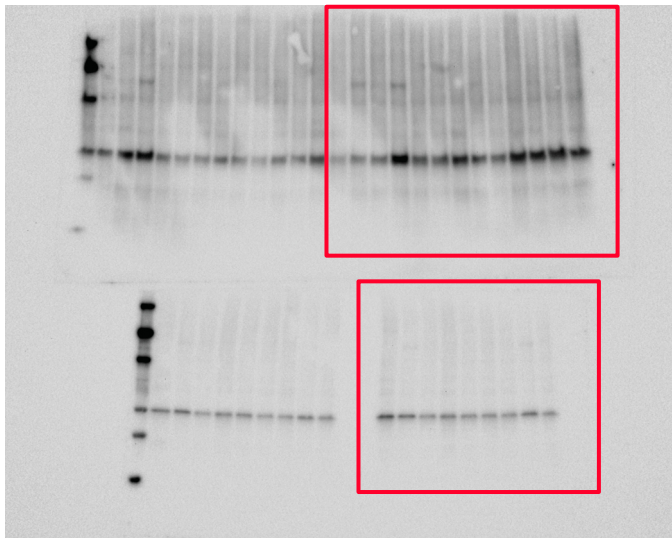
MLKL  
(7G2)



MLKL  
(7G2)



VDAC -  
SDS-PAGE



7G2-  
SDS-PAGE