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mTOR complex-2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum and glucocorticoid induced protein kinase-1 (SGK1)

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Figure 1. mTORC2 deficient MEFs lack SGK1 activity and hydrophobic motif phosphorylation. **(A & B)** Rictor$^{+/+}$ wild type (wt) and Rictor$^{-/-}$ knockout (ko) cells were transfected with the indicated DNA constructs encoding GST-ΔNSGK1 (lacking N-terminal 60 residues encompassing PEST degradation motif). Cells were cultured in the presence of 10% foetal bovine serum to maintain PI 3-kinase pathway activity and lysed 36h post-transfection. SGK1 was affinity-purified on glutathione-Sepharose and subjected to immunoblot analysis with the indicated antibodies and also assayed for activity using Crosstide peptide substrate. The amount of GST-SGK1 immunoprecipitated in each assay was quantified following electrophoresis on an SDS polyacrylamide gel and Coomassie-staining as described in the Materials and Methods. Each bar represents the mean specific activity ± standard error from three different samples, with each sample assayed in duplicate. Cell lysates were also immunoblotted with the indicated antibodies for non-SGK blots. **(C)** As in (**A**) except that cells were transfected with constructs expressing full length SGK1. **(D & E)** as in (**A**) except that mLST8$^{+/+}$ wild type (wt) and mLST8$^{-/-}$ knockout (ko) (**D**) or Sin1$^{+/+}$ wild type (wt) and Sin1$^{-/-}$ knockout (ko) (**E**) MEFs were deployed. Similar results were obtained in three independent experiments.

Figure 2. Dependence of NDRG1 phosphorylation on mTORC2. Immunoblot analysis was undertaken with the indicated antibodies from control wild type (wt) or knockout (ko) MEFs cultured in the presence of 10% foetal bovine serum to maintain PI 3-kinase pathway activity. Cell extracts derived from the skeletal muscle of wild type or SGK1 knockout mice were also analysed. Immunoblots are representative of three different experiments.

Figure 3. Immunoprecipitates mTORC2 phosphorylate SGK1 *in vitro*. **(A)** HEK-293 cell lysates were subjected to immunoprecipitation (IP) with an anti-Rictor or pre-immune IgG antibody. Immunoprecipitates were immunoblotted with the indicated antibodies raised against different mTORC1 and/or mTORC2 components. **(B)** Anti-Rictor or pre-immune IgG immunoprecipitates from HEK-293 cell lysates were incubated with dephosphorylated GST-ΔNSGK1 in the presence of MgATP for 60 min and then subjected to immunoblot analysis with the indicated antibodies. **(C)** As in (**B**) except that samples were assayed in the presence (+) or absence (-) of PDK1. The catalytic activity of GST-SGK1 towards the Crosstide peptide was also measured. The amount of GST-SGK1 present in each assay was quantified following electrophoresis on an SDS polyacrylamide gel and Coomassie-staining as described in the Materials and Methods. Each bar represents the mean specific activity ± standard error from three different samples, with each sample assayed in duplicate. **(D)** As in (**A**) except that cell lysates were derived from mLST8$^{+/+}$ control wild type (wt) or mLST8$^{-/-}$ knockout (ko) MEFs. **(E)** As in (**B**) except that cell lysates were derived from mLST8$^{+/+}$ control wild type (wt) or mLST8$^{-/-}$ knockout (ko) MEFs. Similar results were obtained in three independent experiments.

Figure 4. Immunoprecipitated mTORC1 phosphorylates S6K1 but not SGK1 *in vitro*. **(A)** mTOR was immunoprecipitated from Rictor$^{+/+}$ control wild type (wt) and Rictor$^{-/-}$ knockout (ko) cells that are deficient in mTORC2 but still possess mTORC1. Immunoprecipitates were immunoblotted with the indicated antibodies. (**B & C**) mTOR immunoprecipitates were incubated in the presence (+) or absence (-) of dephosphorylated GST-S6K1 **(B)** or GST-ΔNSGK1 **(C)** in the presence of MgATP for 60 min and then subjected to immunoblot analysis with the indicated antibodies. (**D to F**) 293 cell extracts were subjected to immunoprecipitation with pre-immune (IgG), anti-Raptor or anti-Rictor antibody. The immunoprecipitates were immunoblotted with the indicated antibodies (**D**). The immunoprecipitates were also incubated in the presence (+) or absence (-) of dephosphorylated GST-S6K1 **(E)** or GST-ΔNSGK1 **(F)** in the presence of MgATP for 60 min and then subjected to immunoblot analysis with the indicated antibodies.

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Figure 5. The mTORC1 inhibitor rapamycin does not suppress hydrophobic motif phosphorylation or activation of SGK1 in 3 different cell lines. HEK-293 (A), MCF-7 (**B**) and HeLa (**C**) cells were transfected with a DNA construct encoding GST-SGK1 (full length enzyme). Cells were cultured in the presence of 10% foetal bovine serum in order to maintain PI 3-kinase pathway activity. 36h post-transfection, cells were left treated for 30 min in the presence (+) or absence (-) of 1μM PI-103 or 100 nM Rapamycin. Cells were lysed, SGK1 was affinity-purified on glutathione-Sepharose and either subjected to immunoblot analysis with the indicated antibodies (**A** to **C**) or its catalytic activity assessed employing the Crosstide substrate (**A** only). The amount of GST-SGK1 immunoprecipitated present in each assay was quantified following electrophoresis on an SDS polyacrylamide gel and Coomassie-staining as described in the Materials and Methods. Each bar represents the mean specific activity ± standard error from three different samples, with each sample assayed in duplicate. Cell lysates were also analysed by immunoblotting with the indicated non-SGK antibodies (**A** to **C**). Immunoblots are representative of three different experiments.

Figure 6. Analysis of endogenous phosphorylation of SGK. Lysates from the indicated cell lines generated as described in the legend of Figure 5 were electrophoresed on a 10% polyacrylamide SDS-gels and then subjected to immunoblot analysis with the indicated antibodies. The SantaCruz phospho-Ser422 SGK1 antibody (Catalogue number sc-16745-R) was the same that was utilised in the Hong et al study [46]. Positions of molecular weight markers from Biorad (Catalogue number 161-0373) are shown.

Figure 7. Mechanism of SGK1 activation. In response to insulin and growth factors, PI-3 kinase by an unknown mechanism, stimulates the phosphorylation of SGK1 at its hydrophobic motif via mTORC2. This phosphorylation does not directly activate SGK1, but enables PDK1 to interact with SGK1 through its PIF-pocket docking site, thereby inducing T-loop phosphorylation and activation of SGK1.

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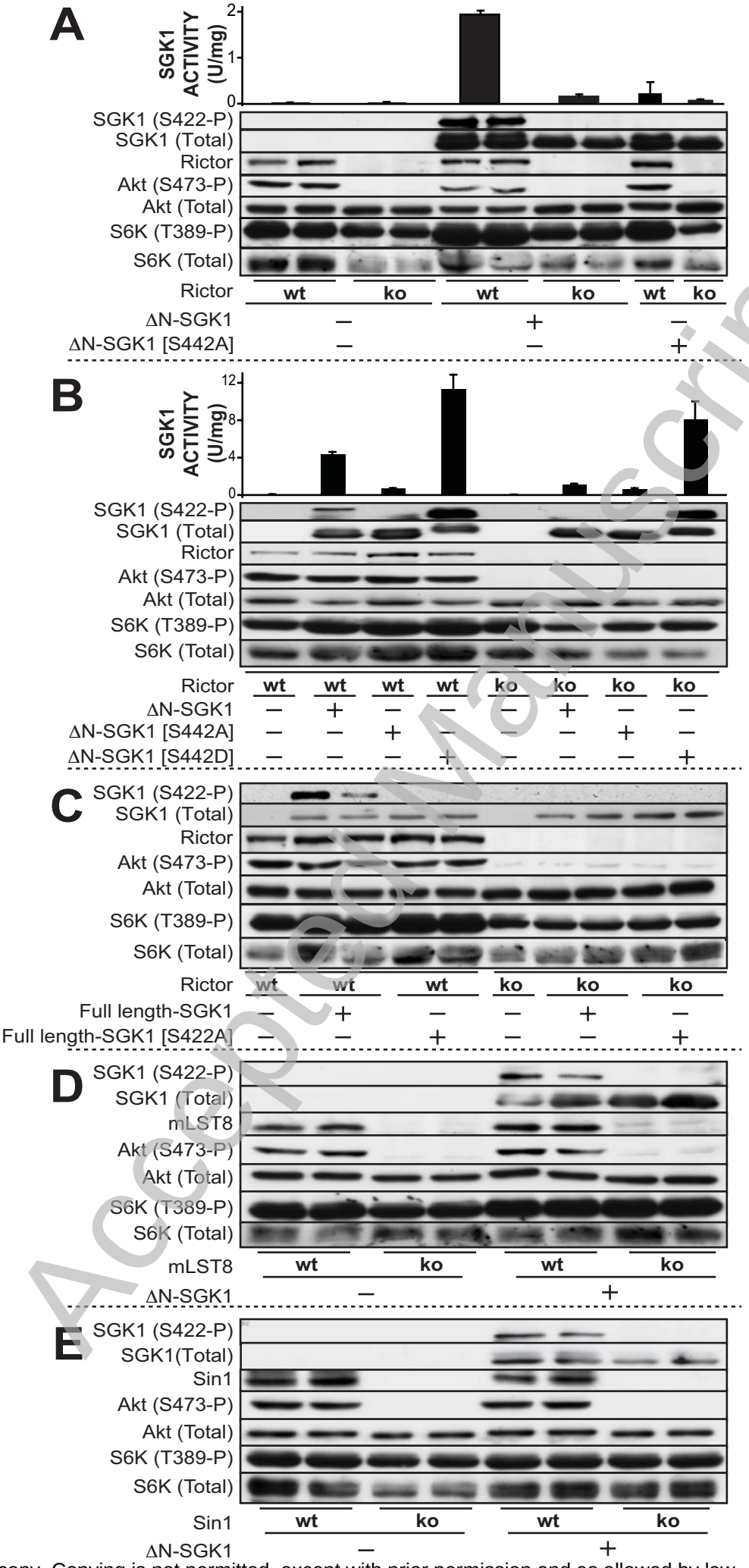

Fig. 1

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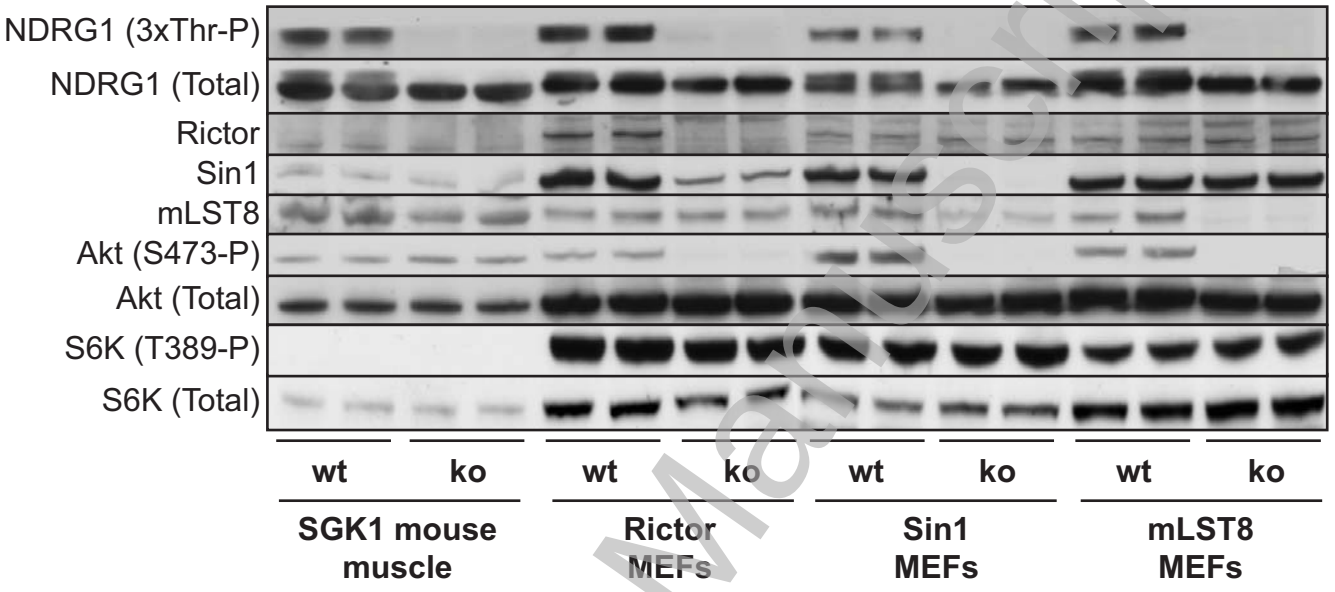

Fig. 2

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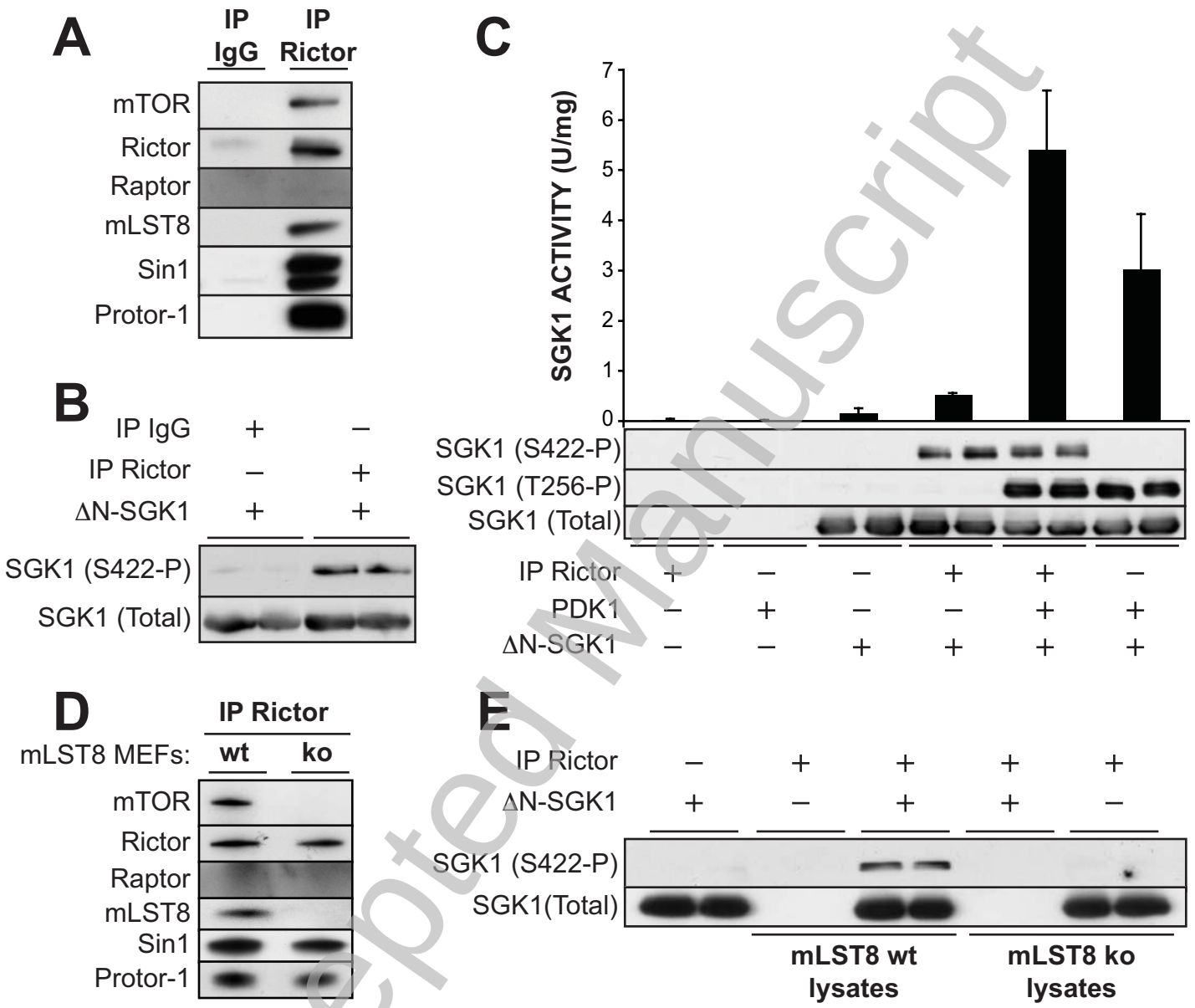

Fig. 3

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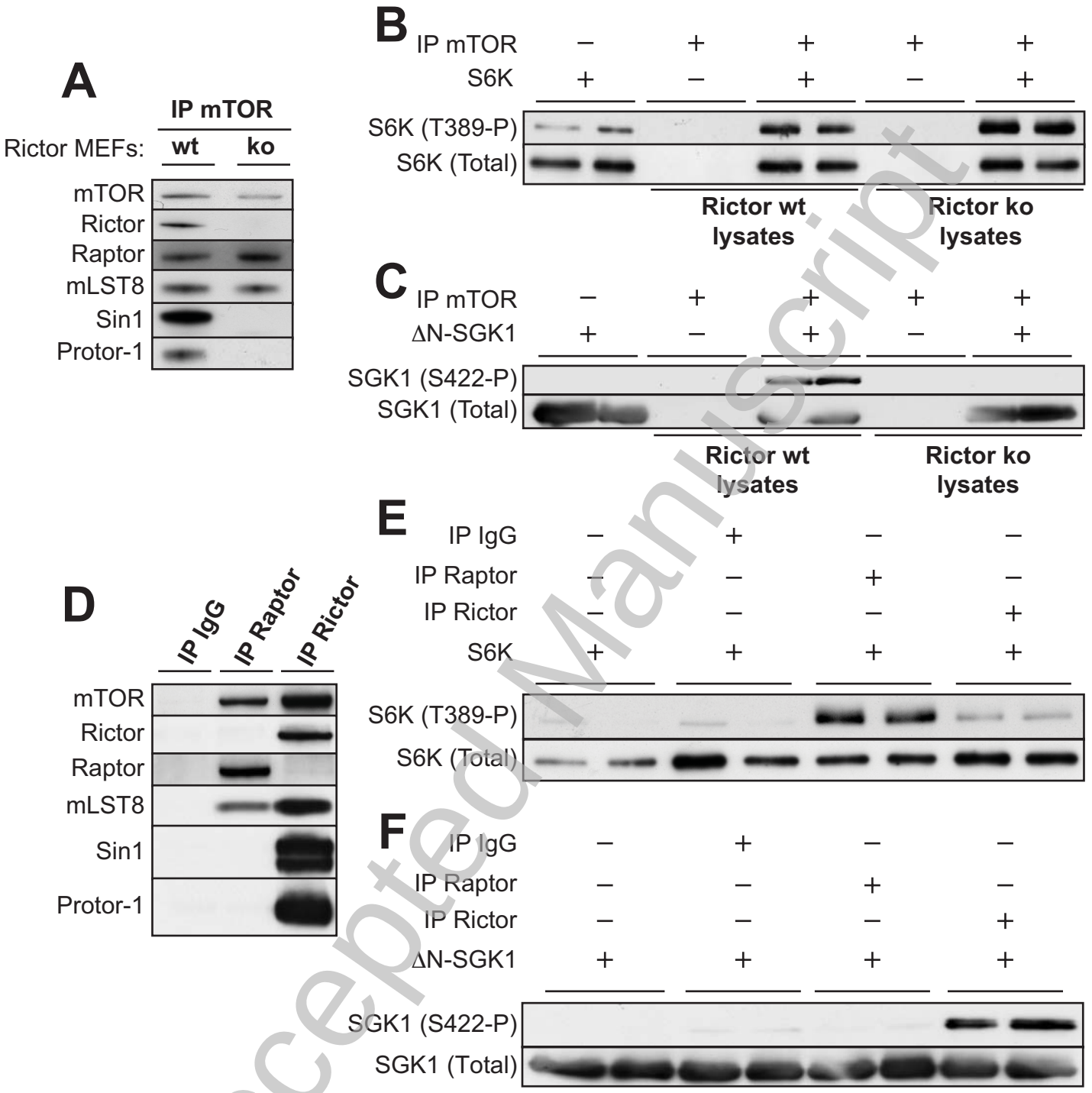

Fig. 4

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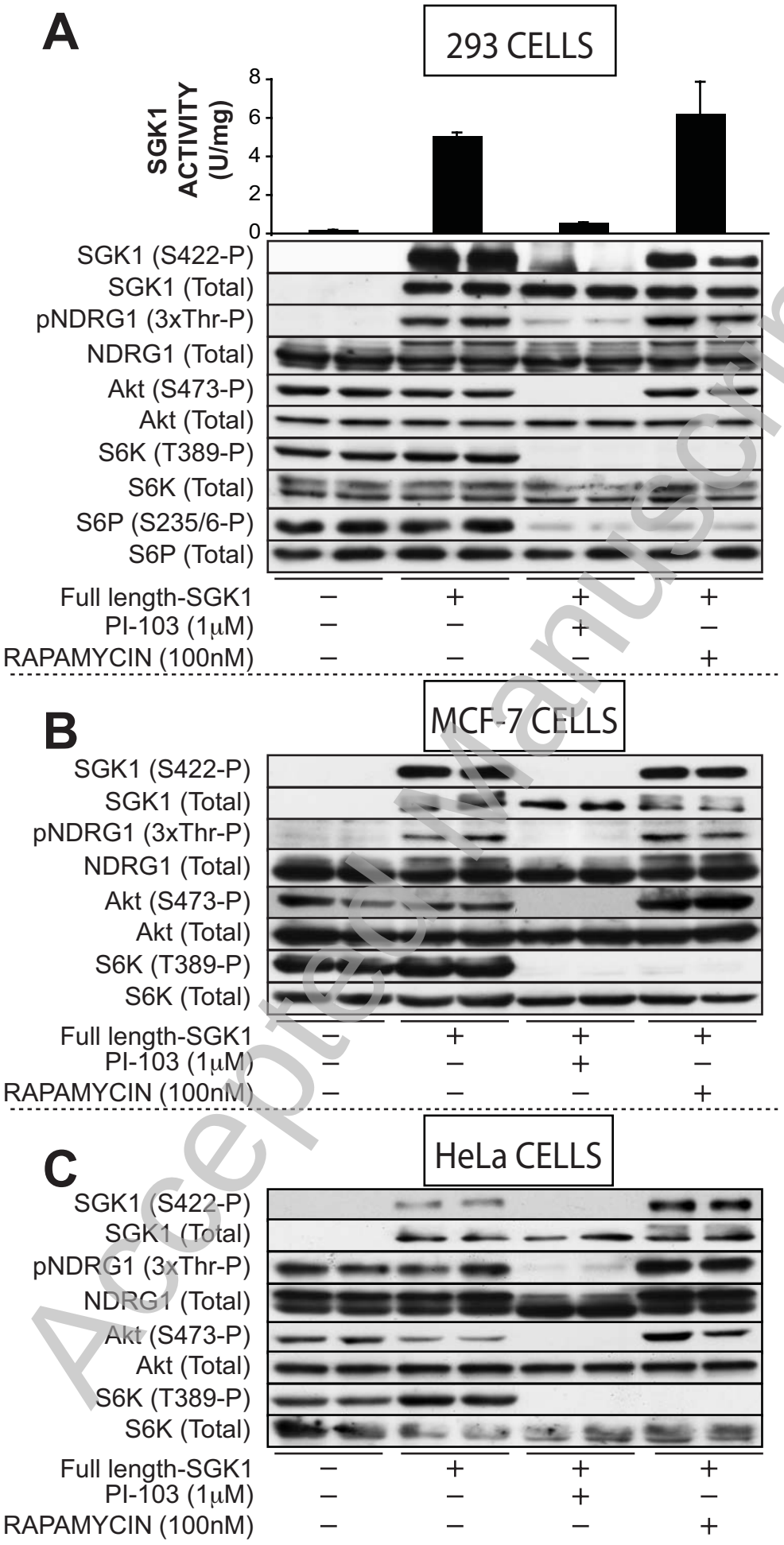

Fig.5

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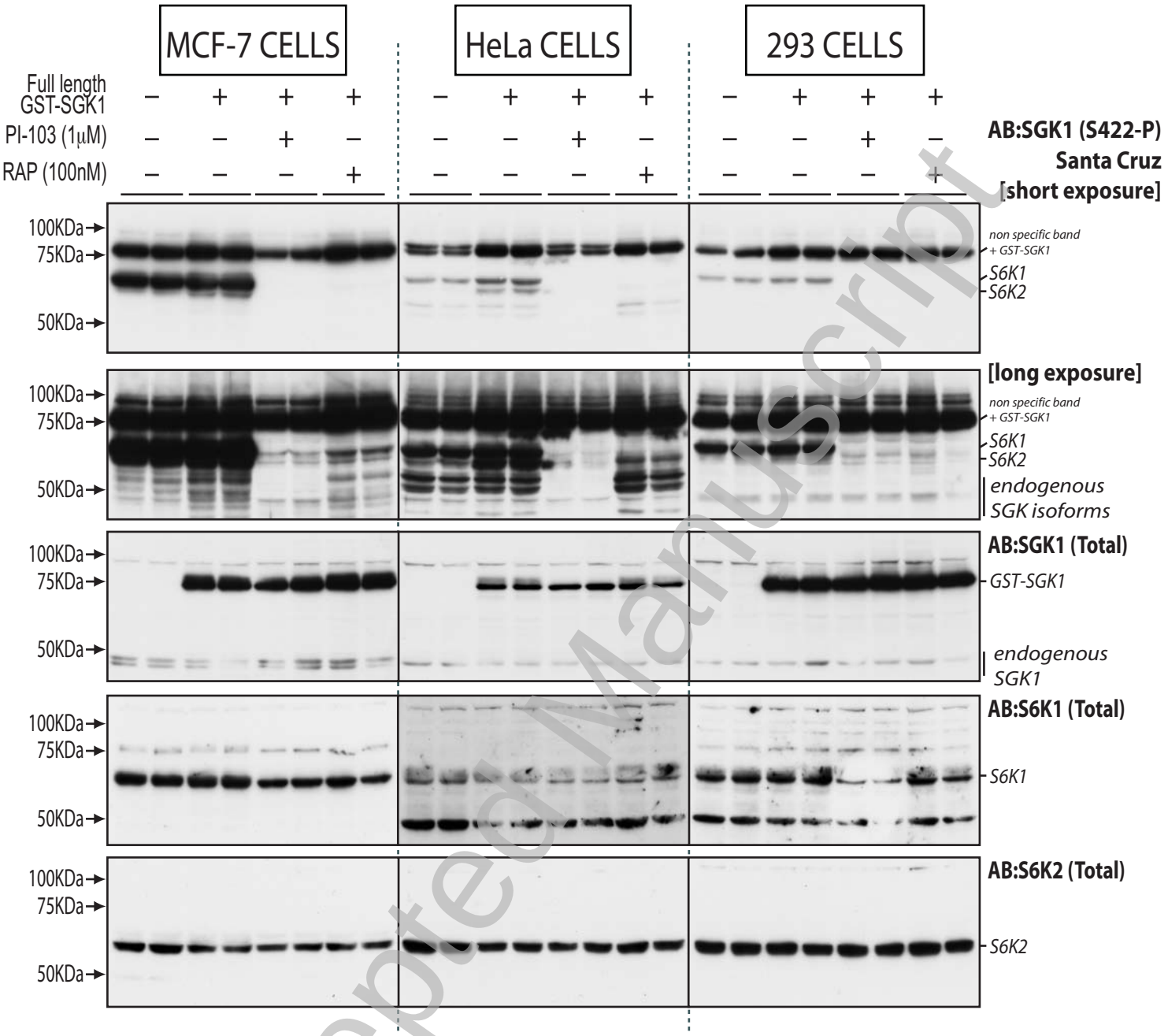

Fig.6

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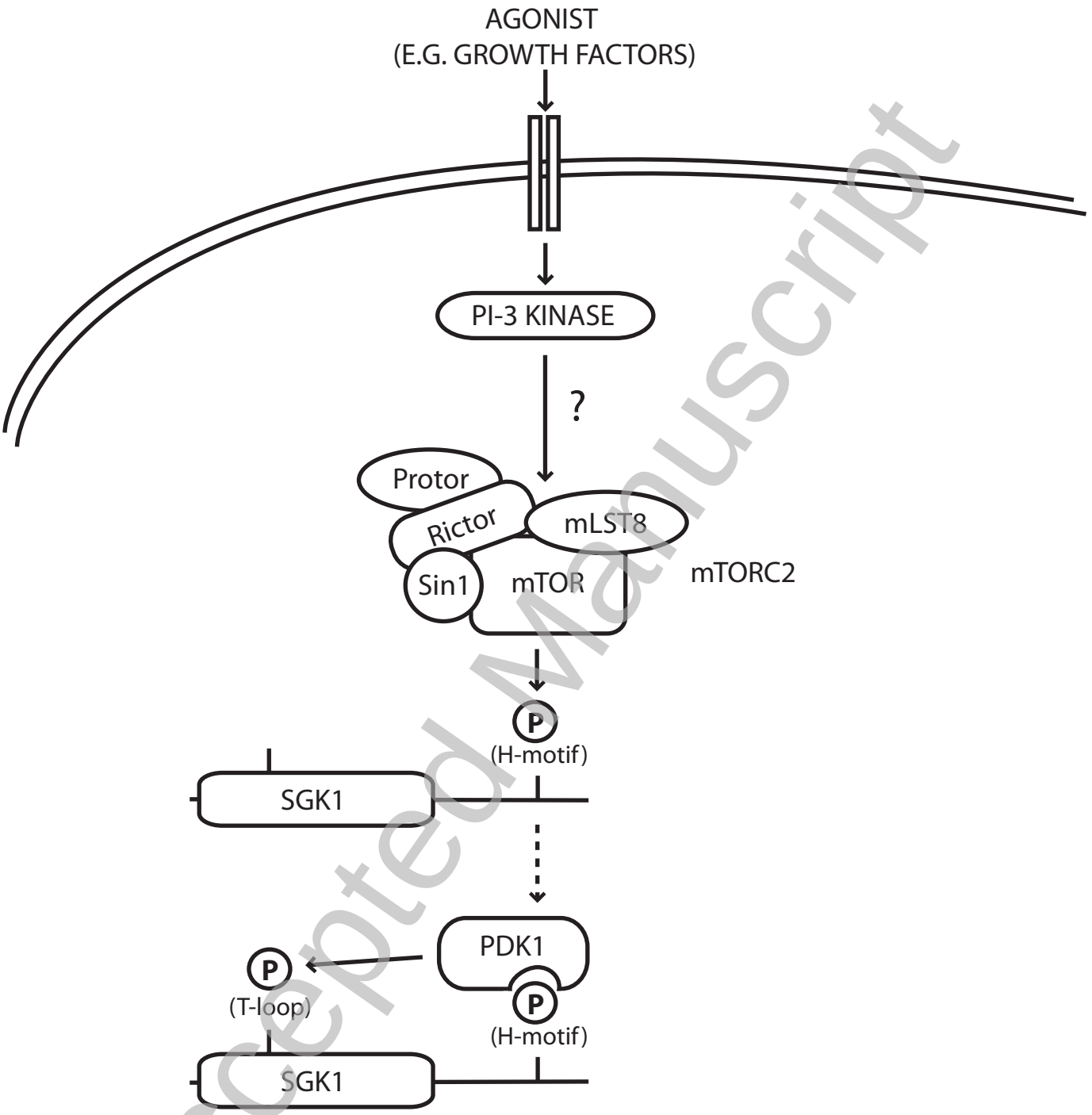

Fig. 7

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