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# Inhibitory Effects of Torin2 on Proximal Tubular Development of the Xenopus laevis Pronephric Kidney

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by

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## ABSTRACT

One of the central components in regulating cell growth and cell cycle progression is the mammalian target of Rapamycin (mTOR) complex. The mTOR complex consists of two distinct forms, mTORC1 and mTORC2. As aberrant mTOR complex activity is a major causative factor of many diseases including Polycystic Kidney Diseases (PKD), mTOR has become a major therapeutic target in the last decade. Rapamycin was the first macrolide drug known to inhibit mTOR activity, specifically inhibiting mTORC1. Recently, small-molecule-inhibitors such as Torin2 have been designed to directly bind the catalytic domain of mTOR in order to inhibit both mTORC1 and mTORC2. Using Rapamycin to block mTORC1, proximal tubular growth of *Xenopus laevis* can be partially abrogated. Here, we investigate whether Torin2 could further inhibit proximal tubular growth by blocking both mTORC1 and mTORC2 activity. Treatment with Torin2 showed greater proximal tubular growth inhibition than Rapamycin, which indicates both mTORC1 and mTORC2 are involved in proximal tubular development.


## INTRODUCTION

Organ development is tightly regulated through a highly specific sequence of signaling events. Improperly regulated signal transduction often results in diseases such as cancer and polycystic kidney disease (PKD). PKD is the most common chronic kidney disease in the US. The etiology of the disease has been linked to genetic mutations that affect kidney developmental signaling pathways [1]. Polycystic kidney disease is characterized by the buildup of fluid filled cysts from renal tubule parenchyma cells [1]. Increased cell proliferation in cysts results in an increase in cyst size, which compresses nephrons, leading to impaired renal function [1]. There is currently no cure for PKD, and the most effective treatments are kidney transplant and dialysis [2].

The proteins responsible for kidney development and renal cyst formation are part of the mammalian target of Rapamycin (mTOR) complex [3]. The mTOR complex consists of two distinct forms, mTORC1 and mTORC2 [4]. In both complexes mTOR is the central serine/threonine protein kinase; however, each complex contains different variations of signal transduction proteins. mTORC1 is composed of regulatory-associated protein of mammalian target of Rapamycin (raptor) and proline-rich Akt substrate, whereas mTORC2 contains Rapamycin-insensitive companion of mTOR (rictor), mammalian stress-activated map kinase-interacting protein (mSin1), and protein observed with rictor 1 and 2 (protor1/2) [4]. Activation of both mTOR complexes occurs in humans suffering from PKD as well as several animal models with PKD phenotypes. The mTORC1 complex activates p70 ribosomal protein S6 kinase (RPS6K) in cystic epithelium which causes an increase in cell size [3]. The mTORC2 complex is involved in the development of PKD by causing an increase in Akt phosphorylation in cystic epithelial cells which promotes cell growth and proliferation [3].

Rapamycin, from which the mTOR pathway was named, is a lipophilic macrolide drug isolated from a strain of *Streptomyces hygroscopicus* that inhibits activation of the mTOR complex (Fig. 1) [5,6]. Rapamycin interacts with protein FKBP12 to inhibit mTOR activity, but the exact inhibitory mechanism of Rapamycin is not completely understood [4]. This drug has been used to reduce renal tubule development and slow cyst growth in several animal models [1]. Interestingly, Rapamycin inhibits the mTORC1 complex more than the mTORC2 complex [4]. Since it does not inhibit mTORC2 as well as mTORC1, new drugs that are similar to Rapamycin (Rapalogs) are being developed to inhibit both complexes of this pathway.

Torins are an example of these newly developed Rapalogs that have greater inhibitory effects on mTOR. Torins are designed to inhibit mTOR activity by binding directly to the mTOR active site, therefore inhibiting mTORC1 and mTORC2 (Fig. 2). Torin1 was the first Torin to be synthesized that had a high selective inhibitory effect on mTOR *in vivo* [7]. However, this drug has poor water solubility, a short half-life, and low oral bioavaliabilty [7]. Scientists have used a medicinal chemistry approach to develop the new drug Torin2 to overcome these negative aspects of Torin1 (Fig. 3) [7,8].

The African clawed frog, *Xenopus laevis*, is a model organism for organogenesis, particularly of the kidney. *Xenopus* are an ideal model organism for kidney development because their kidneys contain a single nephron. *Xenopus* embryos are also very efficient for drug screenings because the drugs can easily be ingested by the embryos through incorporation into the embryo's growth medium. Embryogenesis of *Xenopus* occurs very quickly and is easy to observe; fertilized *Xenopus* eggs develop into swimming tadpoles within 4 days [10]. Kidney development in amphibians like *Xenopus* is unique in that embryos use a sequence of different nephric systems for waste disposal and water homeostasis during development [11]. The three sequential kidney types that arise from the intermediate mesoderm are the pronephros, mesonephros, and the metanephros [12]. The functional pronephros of a *Xenopus* embryo contains three parts: the glomus, the pronephric tubules, and the pronephric duct [12]. The glomus is equivalent to the glomerulus of more complex vertebrates, the pronephric tubules collect waste filtered by the glomus, and the pronephric duct carries waste to the exterior [12].

The pronephros of *Xenopus* embryos become morphologically distinguishable around developmental stage 22 (Fig. 4) [10]. From stages 22-37 the size of the pronephros remains relatively constant in a baseline phase (Fig. 4). Once the embryos reach stage 38 the pronephros

cells begin to rapidly proliferate, and the pronephros undergo expansion; this is known as the growth phase (Fig. 4). The growth phase continues until the embryos reach stage 43 and enter the stationary stage. In the stationary stage, expansion is significantly reduced and terminal differentiation occurs (Fig. 4). Our previous research shows that treating *Xenopus* embryos with Rapamycin reduces their pronephros proximal tubule development by inhibiting mTORC1. In this study, we investigate if Torin2 is able to suppress *Xenopus* pronephros proximal tubule development more effectively by inhibiting both the mTORC1 and mTORC2 complexes.

## MATERIALS AND METHODS

*Embryo Drug Treatment*

*Xenopus* embryos were obtained using *in vitro* fertilization and maintained in 0.1x modified Barth medium and staged using the method according to Nieuwkoop and Faber [13,14]. Rapamycin and Torin2 inhibitory drugs were obtained from Tocris Bioscience [9]. The embryos were treated by dissolving the drugs in DMSO and adding the drugs into the Barth growth medium at stage 34. Rapamycin was used at a 4.0 μM concentration, and the concentration of Torin2 varied.

*Immunohistochemistry and Proliferation Analysis*

For immunohistochemistry on slides embryos were fixed in Dent's fixative (4:1 methanol:DMSO), cleared in 70% ethanol, embedded in paraplast, sectioned at 25 μm, dewaxed, and stained using the 3G8 monoclonal antibody and either the Phospho-S6 (S235/236) or Phospho-AKT (S473) antibodies [11]. The 3G8 antibody was visualized using anti-mouse Alexa-555 and the Phospho-S6 (S235/236)/Phospho-AKT (S473) were visualized using Zenon® 647 (Invitrogen). For whole mount immunostaining the embryos were incubated overnight with the 3G8 monoclonal antibody followed by incubation with a Horseradish peroxidase-coupled

anti-mouse secondary antibody and developed using the ImmPACT DAB kit (Vector Laboratories) [11]. The proliferation analysis was performed as previously described [15]. DAPI was used to label all cell nuclei and the PH3 antibody labels all cells in mitosis.

## RESULTS

*Establishment of an Effective Torin2 Dosage*

The $EC_{50}$ for Torin2 inhibition of mTOR in cell culture is 0.25 nM [9]. Since we wanted to investigate the effects of mTOR inhibition on proximal tubule development in *Xenopus in vitro* an effective dosage concentration needed to be established. An effective dosage concentration is one in which *Xenopus* proximal tubule development is reduced but normal embryonic development is not significantly impaired. In order to establish an effective Torin2 dosage, embryos were treated with varying concentrations of Torin2 from stages 34-42. As expected, Torin2 inhibition of *Xenopus* proximal tubule development is concentration dependent. Treatment with a Torin2 concentration of 0.120 µM reduced the number of proximal tubule cells by approximately 50% and decreased the number of dividing cells by 85% (Fig. 5A and 5B). When embryos were treated with Torin2 concentrations higher than 0.120 µM the embryos were significantly deformed and often disintegrated because of off target effects. Therefore, we chose 0.120 µM as the effective dosage to use in further experiments.

*Determination of mTOR Activity*

Treatment with Torin2 clearly reduces the development of proximal tubules in *Xenopus* embryos; however, we had wanted to confirm that the inhibition of mTOR was actually causing the reduction of proximal tubule development. Immunohistochemistry on slides with antibodies directed against Phospho-S6 (S235/236) and Phospho-AKT (S473) were used to detect the activity of mTORC1 and mTORC2 respectively. Both mTOR complexes are active in the

proximal tubules of untreated embryos, which is indicated by positive red staining for Phospho-S6 and Phospho-AKT (Fig. 6A and 6A'). Treatment of *Xenopus* embryos with Rapamycin inhibits mTORC1 (Fig. 6B'); however, mTORC2 remains active in the proximal tubules (Fig. 6B). Torin2 is able to inhibit the activity of both mTORC1 and mTORC2, which is indicated by the absence of staining for Phospho-S6 and Phospho-Akt respectively (Fig. 6C and 6C'). These results suggest that the inhibition of both mTORC1 and mTORC2 by Torin2 causes the reduction of proximal tubule development in *Xenopus* embryos.

*Reduction of Pronephros Size*

Whole mount immunohistochemistry was used to observe the size of the proximal tubules of the pronephros after treatment with Torin2. At stage 38, the proximal tubules of the Torin2 treated embryos are only slightly smaller than the untreated embryos (Fig. 7A and 7A'). However, at stage 42 the proximal tubules of the untreated embryos appear larger than the Torin2 treated embryos (Fig. 7B and 7B'). Embryos treated with Torin2 do not experience the same proximal tubule expansion as untreated embryos.

*Torin2 Significantly Inhibits Pronephros Proximal Tubule Development Overtime*

Embryos were treated with Rapamycin and Torin2 from stage 34 to 46 and cell proliferation analysis was performed using immunohistochemistry by counting the number of proximal tubule cells of each embryo [15]. The proximal tubule growth of the treated embryos over these stages was compared to the proximal tubule growth of untreated embryos as a control. Proximal tubules of the embryos treated with Torin2 and Rapamycin only expanded by approximately 100 cells over this time period (Fig. 8A). This is a 4-fold decrease in proximal tubule expansion compared to untreated embryos. The number of cells in mitosis is very similar when the embryos are treated with either drug (Fig. 8B). Comparing the percentage of cells that are

currently in mitosis is a better statistic to measure development than the number of cells in mitosis alone. Surprisingly, the proximal tubules of embryos treated with Torin2 have a higher percentage of cells in mitosis than the embryos treated with Rapamycin, but the percentages at each stage are still significantly lower than the untreated embryos (Fig. 8B). The most important statistic in the cell proliferation analysis is the total number of kidney cells at each stage since this statistic is a quantitative representation of kidney size. The number of total cells when treated with Torin2 is 20% less than when the embryos are treated with Rapamycin (Fig 8A). The more effective expansion inhibition by Torin2 suggests that the mTORC2 complex is also involved in proximal tubule expansion since Torin2 is able to inhibit this complex in addition to mTORC1, whereas Rapamycin is only able to inhibit the mTORC1 complex. The mTORC2 complex is most likely involved in early proximal tubule cell proliferation and is thus why there is a gap in the number of proximal tubule cells at stage 38 between the Rapamycin and Torin2 treated embryos (Fig. 8A). These findings provide evidence for Torin2 being a better inhibitor of mTOR *in vivo* and thus more effective at reducing *Xenopus* pronephros proximal tubule development.

## DISCUSSION

mTOR has been shown to have a critical role in normal kidney development and aberrant mTOR activity can lead to renal diseases such as Polycystic Kidney Disease. Treatment with Rapamycin has been able to inhibit mTOR activity and reduce kidney proximal tubule development in model organisms such as *Xenopus*. Although Rapamycin is able to inhibit mTOR activity it is only able to inhibit the mTORC1 complex whereas the mTORC2 complex remains active (Fig. 6B and 6B'). This study investigated how the Rapalog Torin2 inhibits *Xenopus* pronephros proximal tubule development compared to Rapamycin. Treating *Xenopus* embryos with Torin2 inhibits both TORC1 and TORC2 activity in the pronephros proximal

tubules (Fig. 6C and 6C'). We were able to visualize smaller pronephros proximal tubules in Torin2 treated embryos through whole mount immunohistochemistry (Fig. 7B and 7B'). We were also able to quantitatively measure the effects of Torin2 on proximal tubule development by using the cell proliferation analysis technique established by Romaker et al. [15]. By inhibiting both mTOR complexes, Torin2 reduces pronephros proximal tubule expansion more effectively than Rapamycin (Fig. 8A). Although there was a higher percentage of cells in mitosis in the embryos treated with Torin2 than Rapamycin, the total number of cells in Torin2 treated embryos was lower (Fig. 8C and 8B). These results suggests that Torin2 could potentially be a better pharmacological therapeutic for renal developmental diseases such as Polycystic Kidney Disease.

In order to further investigate the effects of Torin2 on kidney development, it would be interesting to reproduce similar experiments using the murine system. These results would provide a better indication of how Torin2 would affect human kidney development since mice have similar and more complex kidneys than *Xenopus*. It would also be interesting to examine the effects of Torin2 treatment on cyst development in mice expressing the PKD phenotype. Previous studies have shown that treating PKD mice with Rapamycin is able to reduce kidney cyst size and reduce the kidney mass back to wild-type levels [16]. However, Rapamycin treatment was not able to reduce the number of cysts in the kidneys [16]. Since Torin2 is able to inhibit both mTOR complexes maybe it could potentially reduce the number of cysts in addition to cyst size in PKD mice. Expanding this study to the murine system would provide great insight on the potential therapeutic effects of Torin2 for PKD patients.

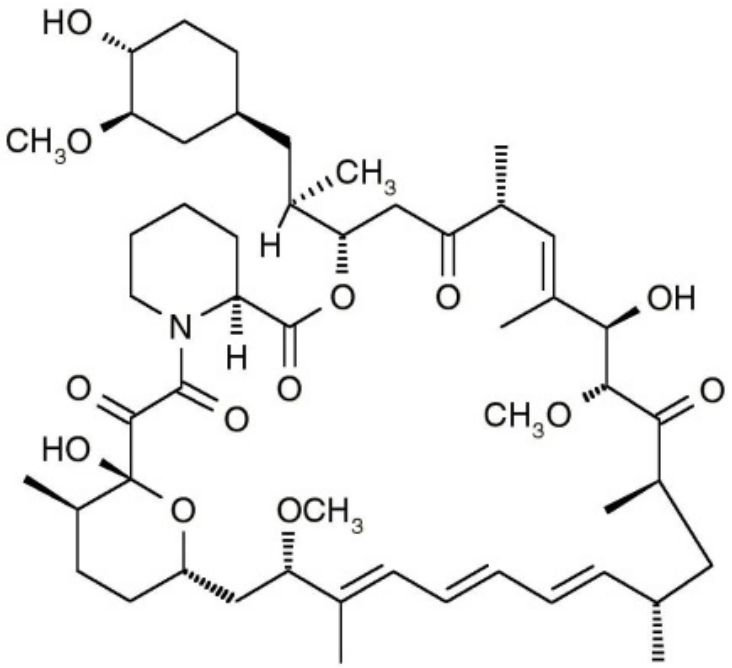


**Figure 1. Chemical Structure of Rapamycin.** Rapamycin interacts with the protein FKBP12 to inhibit the mTORC1 complex. The exact inhibitory mechanism is not completely understood, and Rapamycin is unable to inhibit mTORC2 activity.

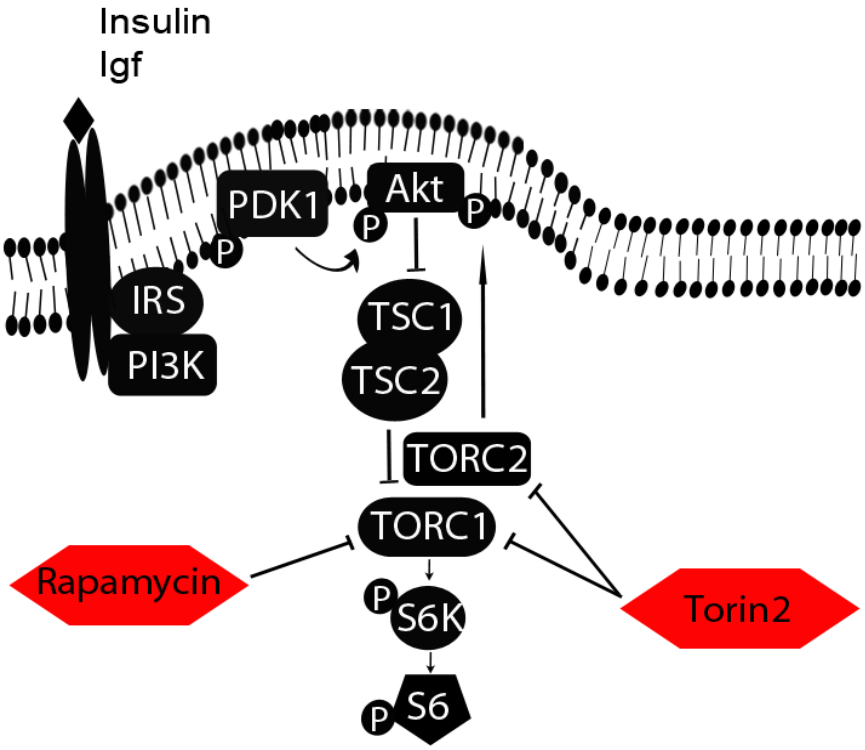


**Figure 2. Schematic Diagram of mTOR Inhibition by Rapamycin and Torin2.** Insulin and IgF are two activators of the mTOR pathway in *Xenopus*. Rapamycin inhibits the mTORC1 complex by interacting with FKBP12 which inhibits the phosphorylation of S6 thereby prohibiting the cell from increasing in size. Torin2 binds directly to the mTOR active site to inhibit the mTORC1 and mTORC2 complexes. Inhibition of mTORC2 prevents the phosphorylation of Akt which prevents cell growth and proliferation.

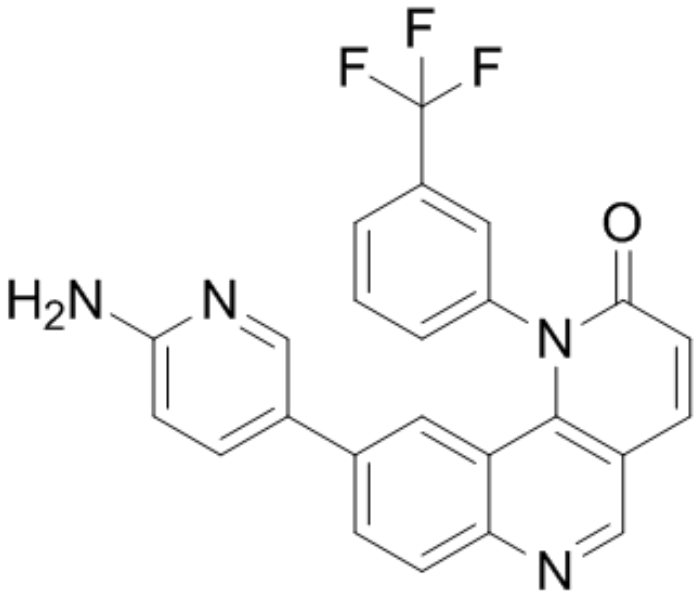


**Figure 3. Chemical Structure of Torin2.** Torin2 is a Rapalog that is able to inhibit mTOR by binding directly to the active site therefore inhibiting both mTORC1 and mTORC2 activity.

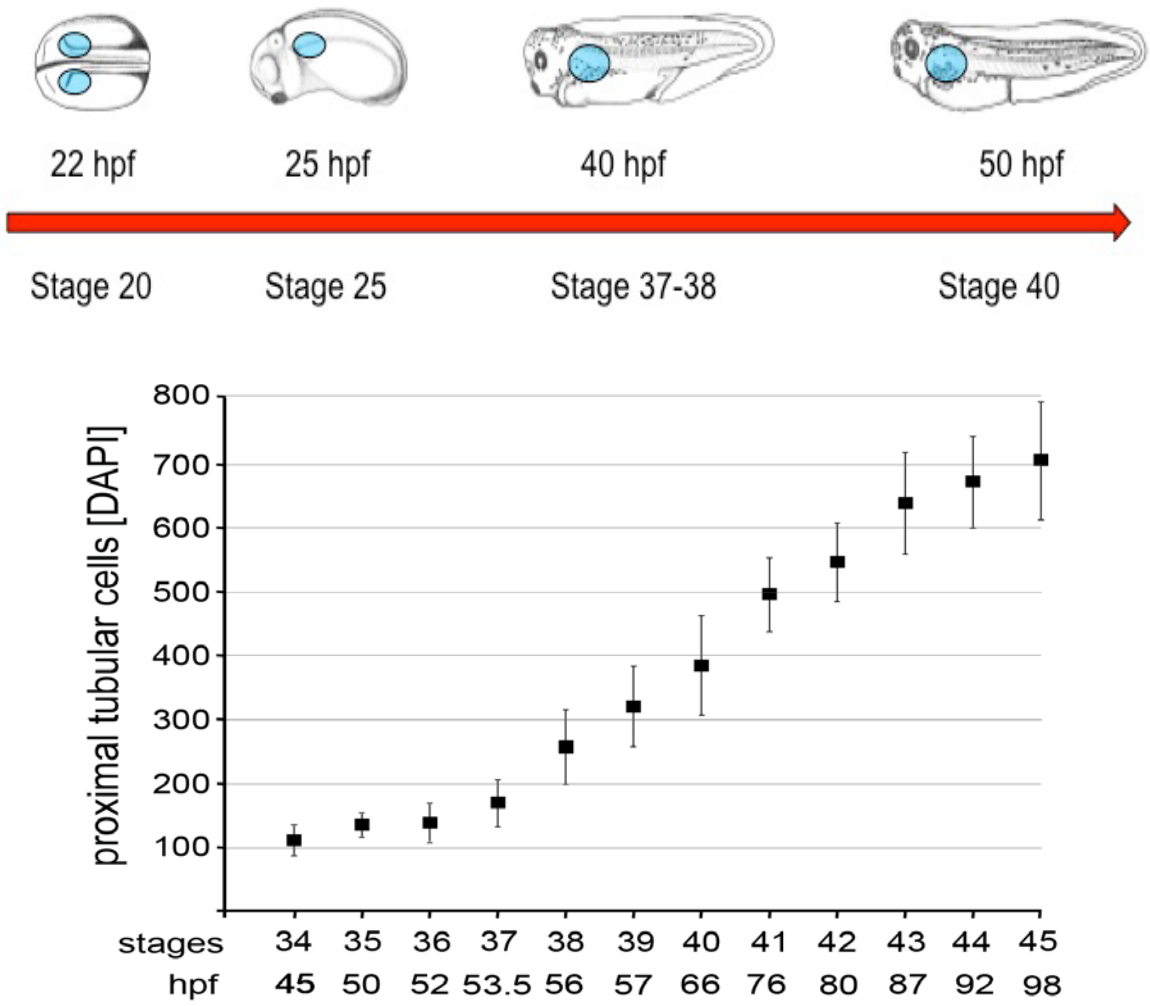


**Figure 4. Differentiation of *Xenopus laevis* pronephros.** The pronephros proximal tubule development can be divided into 3 distinct phases: stages 22-37: baseline phase, stages 38-42: growth phase, stages 43-45: stationary phase. Hpf indicates the hours post fertilization.

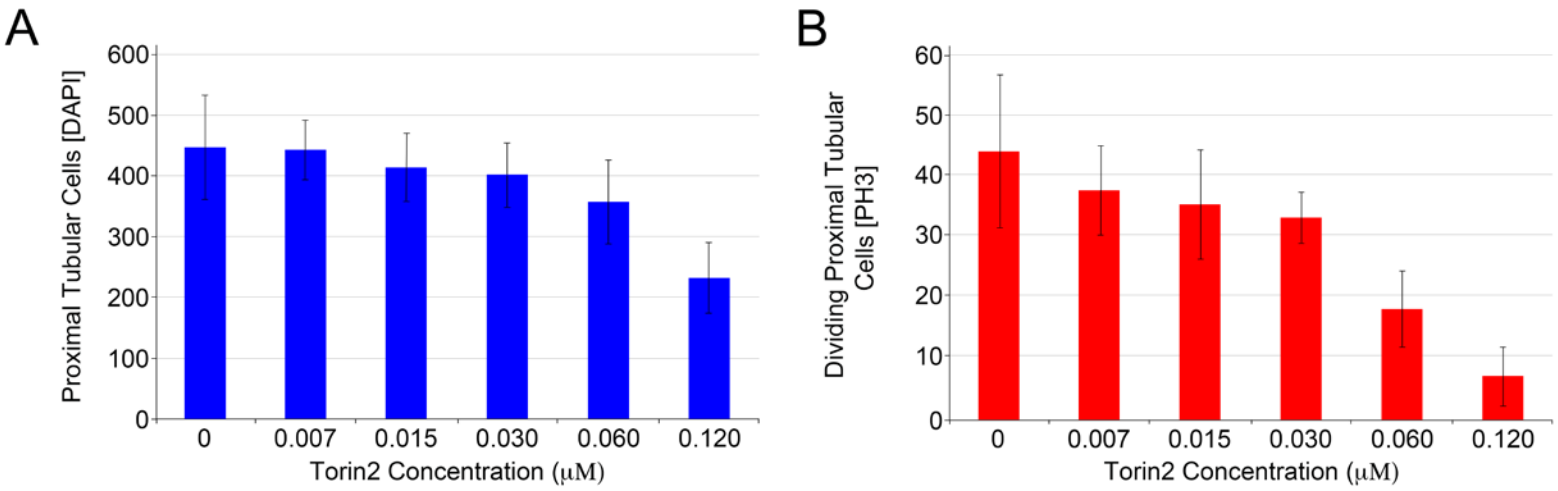


**Figure 5. Effectiveness of Torin2 is Concentration Dependent.** (A) Total number of pronephros proximal tubule cells. (B) Number of pronephros proximal tubule cells in mitosis. The growth of the proximal tubules was measured after treatment with varying concentrations of Torin2. Torin2 inhibits proximal tubule growth in a concentration dependent manner. Treatment with 0.120 μM Torin2 inhibits approximately 50% of tubule growth. This concentration is an effective treatment dose because it inhibits tubule growth without significantly impacting normal embryonic development.

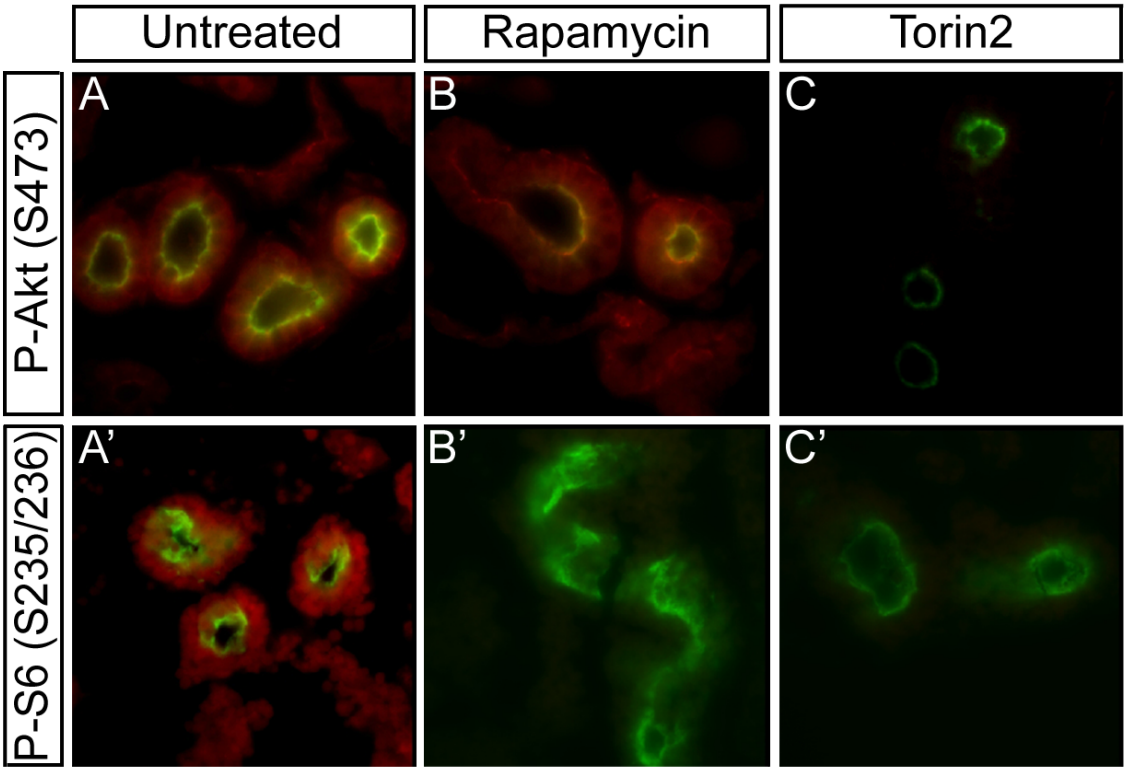


**Figure 6. Rapamycin Inhibits mTORC1 and Torin2 Inhibits mTORC1 and mTORC2.** Immunohistochemistry on slides with antibodies directed against Phospho-Akt (mTORC2) and Phospho-S6 (mTORC1) were used. Untreated embryos have active mTORC1 and mTORC2 (A and A'). mTORC2 remains active (B) when embryos are treated with Rapamycin however mTORC1 is inactivated (B'). Torin2 is able to inhibit both mTORC1 and mTORC2 activity (C and C').

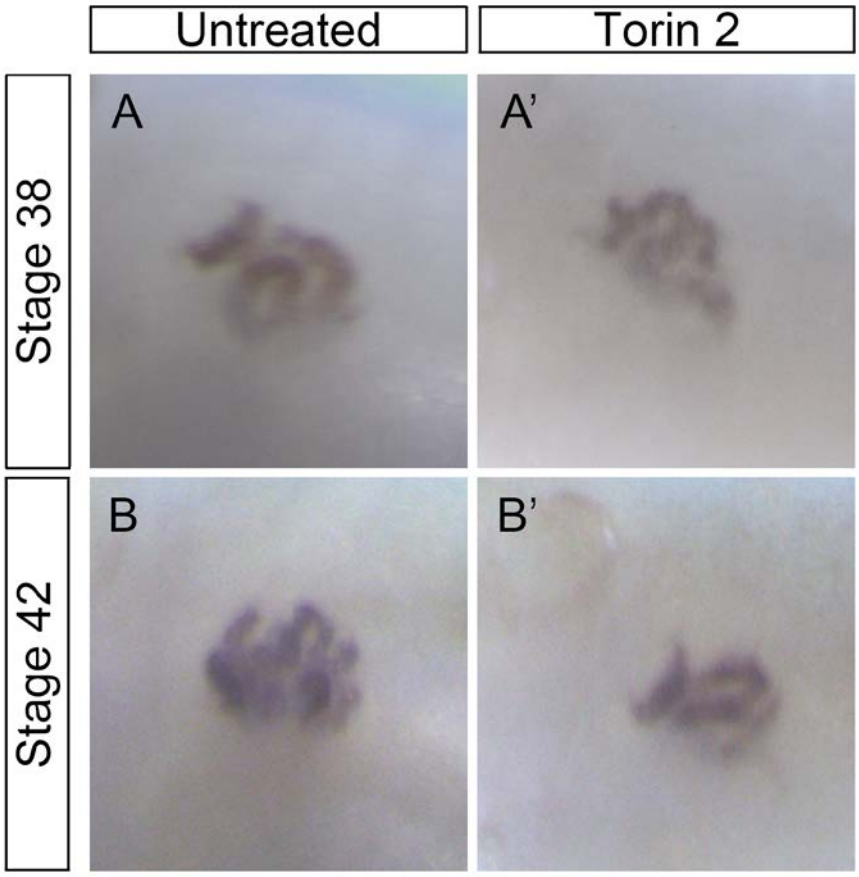


**Figure 7. Pronephros Size After Torin2 Treatment.** Untreated embryos have significant proximal tubule expansion from stage 38 to 42 (A and B) while Torin2 prevents this expansion (A' and B').

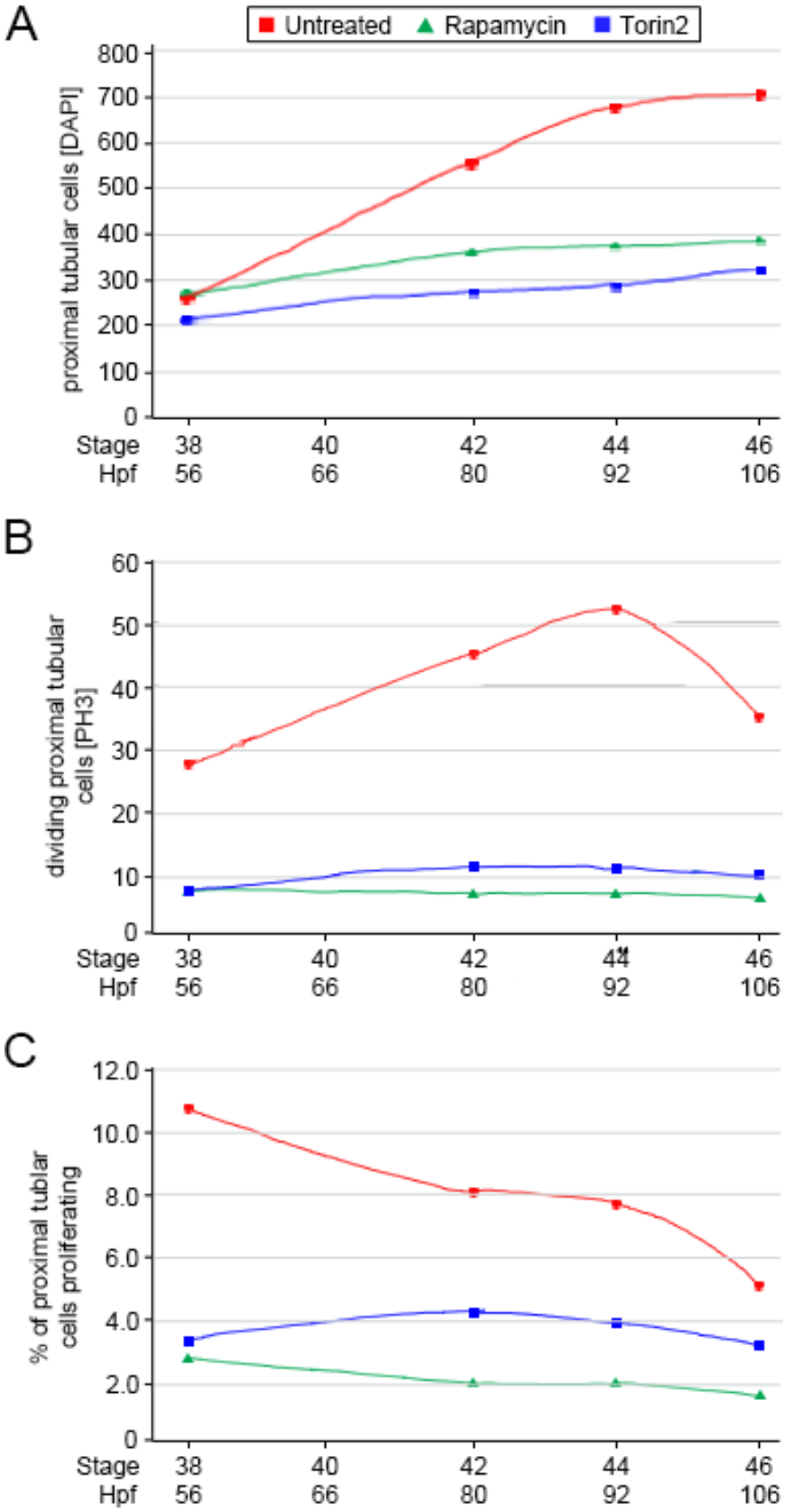


**Figure 8. Cell Proliferation Analysis of Pronephros Proximal Tubules After Drug Treatment.** The proximal tubule growth of treated embryos from stages 38 to 46 was compared to the proximal tubule growth of untreated embryos as a control. Proximal tubules of the embryos treated with Torin2 only expanded by approximately 100 cells over this time period (A). This is a 4-fold decrease in proximal tubule expansion compared to untreated embryos. The number of proximal tubular cells dividing over these stages (B) and percentage of cells dividing (C) were significantly less than untreated embryos. Torin2 reduces proximal tubule expansion more effectively than Rapamycin (A). The more effective expansion inhibition by Torin2 suggests that the TORC2 complex is also involved in proximal tubule expansion since Torin2 is able to inhibit this complex in addition to TORC1, where Rapamycin is only able to inhibit the TORC1 complex.

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