

SHORT COMMUNICATION

The kinetics of ER fusion protein activation *in vivo*

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Reversibly switchable proteins are powerful tools with which to explore protein function *in vitro* and *in vivo*. For example, the activity of many proteins fused to the hormone-binding domain of the modified oestrogen receptor (ER^{TAM}) can be regulated by provision or removal of 4-hydroxytamoxifen (4-OHT). Despite the widespread use of ER^{TAM} fusions *in vivo*, inadequate data are available as to the most efficacious routes for systemic tamoxifen delivery. In this study, we have used two well-characterized ER^{TAM} fusion proteins, both reversibly activated by 4-OHT, to compare the effectiveness and kinetics of 4-OHT delivery in mice *in vivo* by either tamoxifen in food or by intraperitoneal injection. Our data indicate that dietary tamoxifen offers an effective, facile and ethically preferable means for long-term activation of ER^{TAM} fusion proteins *in vivo*.

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INTRODUCTION

In classical mouse genetics, the function of a specific gene is irreversibly mutated or deleted in the germ-line and the phenotype that manifests is an amalgam of both, the direct consequences of the mutation together with the extent to which those direct consequences drive compensatory adaptation during development. Such developmental compensation, together with the embryonic lethality associated with other mutations, severely limits the information that such ‘germ-line’ genetics can provide on the roles played by genes in adult tissues. The inability to reverse the germ-line alterations of genes in classical genetics further limits determination of adult gene function. Such considerations have fostered the idea of using reversibly switchable genetic technologies, whereby wild-type gene functions may be reversibly toggled on and off, at will, in adult mice, either systemically or tissue by tissue.

When fused to a heterologous protein, the modified oestrogen receptor ligand-binding domain (ER^{TAM}) may be used to render that protein rapidly and reversibly ectopically regulatable by the ER^{TAM} ligand 4-hydroxytamoxifen (4-OHT), see <http://www.picard.ch/downloads> for annotated list of proteins that have successfully been regulated by fusion with a steroid-binding domain. Most notably, ER^{TAM} fusions with various oncoproteins and tumour suppressor proteins have been successfully employed in transgenic and knock-in mouse cancer models to ascertain the roles of such effectors in both tumour initiation and maintenance.^{1–4} However, the efficiency of tamoxifen delivery and activation of ER^{TAM} fusion proteins has hitherto only been assessed for ‘hit-and-run’ activation functions, such as activation of Cre-ER^{TAM} by determining the extent of recombination of specific floxed alleles.^{8–10} However, such acute studies offer little guidance as to the practical utility of tamoxifen administration in sustaining the activities of ER^{TAM} fusion proteins over protracted periods *in vivo*, as is required for modelling chronic pathologies such as cancer that typically requires long-term administration of ER^{TAM} ligand to sustain activities of oncogenic ER^{TAM} fusion proteins. For such purposes, 4-OHT is prohibitively expensive and, consequently, most *in vivo* studies instead use tamoxifen, which is cheaper and is

readily metabolized by the liver into the active 4-OHT metabolite by the cytochrome P450 CYP2D6 and CYP3A4 isoforms.⁵ Unfortunately, tamoxifen has a relatively short half-life *in vivo* (about 16 h)⁶ and is poorly soluble in aqueous preparations so, to date, the most consistent results for sustained activation of ER^{TAM} fusion proteins have been obtained through daily intraperitoneal injection (i.p.) of tamoxifen dispersed in vegetable oil.⁷ However, administration via this route is both labour-intensive and ethically debatable because it can result in sterile peritonitis due to the accumulation of oil. Indeed, six weeks of daily injections is the maximum that can be realistically tolerated, therefore, extended treatment with tamoxifen is usually impractical. Despite the widespread use of ER^{TAM} fusions *in vivo*, scant data are available on the most efficacious routes for long-term tamoxifen delivery. We therefore sought to ascertain the kinetics of tamoxifen-induced activation of ER^{TAM}-fusion proteins in response to various routes of tamoxifen administration and to investigate more sustainable tamoxifen delivery methods for long-term analysis.

RESULTS AND DISCUSSION

We employed two well-characterized mouse models whose biologies are dependent upon activities of ER^{TAM} fusion proteins. First, the *pIns-MycER^{TAM}* mouse, in which transgenic overexpression of the 4-OHT-dependent allele of the c-Myc oncoprotein, MycER^{TAM}, is driven from the rat insulin promoter in pancreatic β-cells. Administration of tamoxifen to such animals activates oncogenic Myc, triggering widespread Myc-induced β-cell apoptosis and islet involution. However, co-expression of the anti-apoptotic protein Bcl-x_L in β-cells blocks Myc-induced apoptosis, whereupon Myc activation drives and maintains synchronous, rapid, persistent and progressive β-cell expansion accompanied by angiogenesis, inflammation, invasion and tumorigenesis.¹¹ Subsequent de-activation of MycER^{TAM} triggers complete regression of the β-cell tumours. Second, we used *Trp53^{tm1Gev}* mice (hereafter called *p53^{Ki/Ki}*), in which both copies of endogenous *p53* are replaced by a gene encoding the 4-OHT-dependent *p53* allele *p53ER^{TAM}*.⁴ In these animals, *p53* function is continuously

dependent upon the provision of 4-OHT ligand. Hence, in the absence of tamoxifen, $p53^{KI/KI}$ mice are effectively p53 null and radiosensitive tissues, such as thymus, spleen and intestinal epithelium, exhibit significant refractoriness to radiation-induced apoptosis, as they do in $p53^{-/-}$ mice. However, p53-dependent radiosensitivity of $p53^{KI/KI}$ tissues is rapidly restored upon systemic administration of tamoxifen.⁴

To assess whether dietary tamoxifen is a feasible means for regulating ER^{TAM} protein activity *in vivo*, we first assessed the dynamics of oncogenic MycER^{TAM} activation in *plns-MycER^{TAM}; RIP7-Bcl-x_L* mice (also known as *Tg(Ins-BCL2L1)2Ksp;Tg(Ins-MYC/Er)1Gev* mice).¹¹ Myc-driven pancreatic insulinomas were induced in 8- to 15-week-old *plns-MycER^{TAM};RIP7-Bcl-x_L* ($n \geq 3$) mice by either daily i.p. of 0.8 mg tamoxifen in oil or dietary tamoxifen for 7 days. β -cell proliferation in pancreata was then assessed by immunohistochemical staining for the proliferative marker Ki67. Control *RIP7-Bcl-x_L* mice exhibited little β -cell proliferation (< 0.4 Ki67 positive cells per unit area) irrespective of tamoxifen delivery or route of administration. By contrast, administration of tamoxifen to *plns-MycER^{TAM};RIP7-Bcl-x_L* mice, by either injection or diet, elicited similarly dramatic levels of β -cell proliferation ($P = 0.85$, Figure 1), indicating that MycER^{TAM}-activating doses of tamoxifen are achievable by both administration methods. Hence, administration of tamoxifen through diet is an effective means for achieving ER^{TAM}-activating levels of 4-OHT in tissues *in vivo*.

To establish the kinetics and persistence of tamoxifen-dependent activation of ER^{TAM} proteins in tissues *in vivo*, we used the $p53^{KI/KI}$ mouse model, assessing 4-OHT-dependent p53 functionality using responses of radiosensitive tissues to pathological doses of γ -irradiation. Tamoxifen was administered to 8–12-week-old homozygous $p53^{KI/KI}$ mice (and to $p53^{+/+}$ and $p53^{-/-}$ controls) by either (i) single intraperitoneal bolus of 1 mg tamoxifen dissolved in oil or by feeding mice with tamoxifen-impregnated food for (ii) 24 h or (iii) 7 days. Mice were then exposed to 2.5 Gy whole body γ -irradiation (Cs source) at various times after bolus tamoxifen administration or after removal from tamoxifenized food, and the thymus and spleen were collected 4 h later. p53 functionality was assessed by quantifying apoptosis in the thymus (immunohistochemical staining for cleaved caspase-3) (Figures 2a–c) and by assaying levels of $p21^{cip1}$ expression in the spleen (Supplementary Figure 1).

As expected, untreated $p53^{+/+}$ control mice exhibited high levels of both apoptosis and $p21^{cip1}$ expression following irradiation whereas non-tamoxifen-treated $p53^{KI/KI}$ mice (as well as $p53^{-/-}$ controls), exhibited little (Supplementary Figure 2). Significant apoptosis and $p21^{cip1}$ expression were evident in the radiosensitive tissues of $p53^{KI/KI}$ within only 2 h of i.p. administration of 1 mg tamoxifen, peaking around 4 h post injection and then falling back to background levels between 24–48 h post treatment. Hence, tamoxifen administered i.p. rapidly and systemically restores functionality to p53ER^{TAM} tissues *in vivo*. Attenuation of radiosensitivity was more rapid (50% reduction in $p21^{cip1}$ expression by 8 h post irradiation) in mice given a single i.p. injection of a reduced (0.8 mg) tamoxifen dose (estimated to be equivalent to the dose received via chow, see below) whereas a higher dose (4 mg) elicited a more sustained p53ER^{TAM} functionality (more than 80% of peak $p21^{cip1}$ expression was maintained to 8 h post irradiation). However, higher doses of tamoxifen did not increase the extent of $p21^{cip1}$ activation at 2 h post irradiation (Supplementary Figure 3A), indicating that the level of p53 activation is directly dependent on tamoxifen availability and that tamoxifen doses via i.p. greater than 0.8 mg tamoxifen are fully saturating for p53ER^{TAM} *in vivo* and that higher doses merely confer more prolonged ER^{TAM} fusion protein activity.

We next investigated the kinetics of ER^{TAM} activation when delivering tamoxifen through food. $p53^{KI/KI}$ mice exposed to tamoxifen-containing food for 24 h exhibited no measurable increase in radiosensitivity. This is most likely due to the fact that over the first 24-h period, mice only ate on average 2.3 g of tamoxifen-containing food ($n = 14$, range 1–3 g), which is around half of their normal daily intake and, consequently, exhibited on average 8.5% weight loss ($n = 14$, range 0–19%). We guess that the level of tamoxifen in the animals is insufficient to activate p53ER^{TAM} and, for this reason, dietary tamoxifen is unsuitable for studies requiring acute activation of ER^{TAM} proteins. However, tissues of $p53^{KI/KI}$ mice exposed to tamoxifen-containing food for 7 days was sufficient to render tissues fully radiosensitive, as evidenced by extensive radiation-induced apoptosis and $p21^{cip1}$ expression that then fell to background levels by 16–24 h of surcease of dietary tamoxifen (Figure 2 and Supplementary Figure 1C). The actual DNA damage induced by γ -irradiation, as assessed by phospho-Histone H2A.X staining, was comparable in

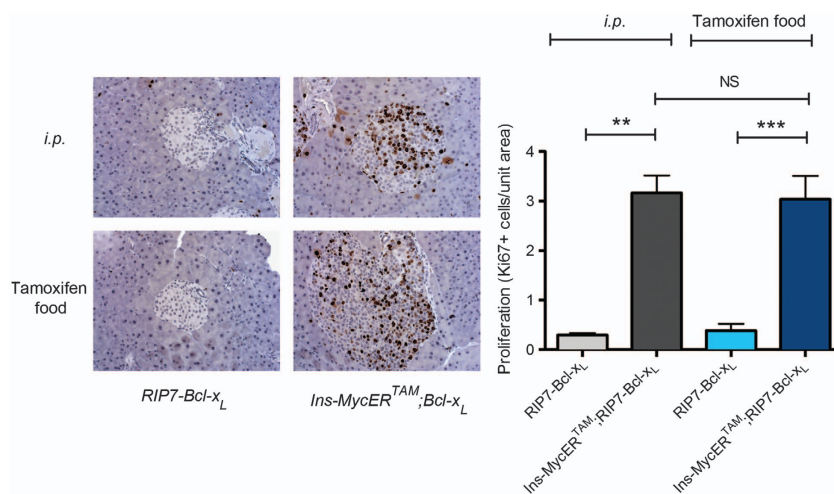


Figure 1. Persistence of c-Myc functionality in *plns-MycER^{TAM};RIP7-Bcl-x_L* mice after different routes of tamoxifen administration (a) Representative images of pancreatic islets stained for the Ki67 proliferative marker from *plns-MycER^{TAM};RIP7-Bcl-x_L* and *RIP7-Bcl-x_L* controls showing enlarged highly proliferative hyperplastic islets. The histogram depicts quantification of Ki67 immunohistochemical staining. Quantification was performed on three islets per mouse and area measured; Ki67 positive stained cells were counted per islet and ratio per unit area was calculated. No significant difference was calculated (t-test, $P = 0.85$) between *plns-MycER^{TAM};RIP7-Bcl-x_L* mice treated with either injection or dietary tamoxifen. Mean and s.e.m. shown, $***P < 0.001$, $**P < 0.01$. Primary antibody: anti-Ki67 (Thermo Scientific, Fremont, CA, USA; clone: SP6; 1:200).

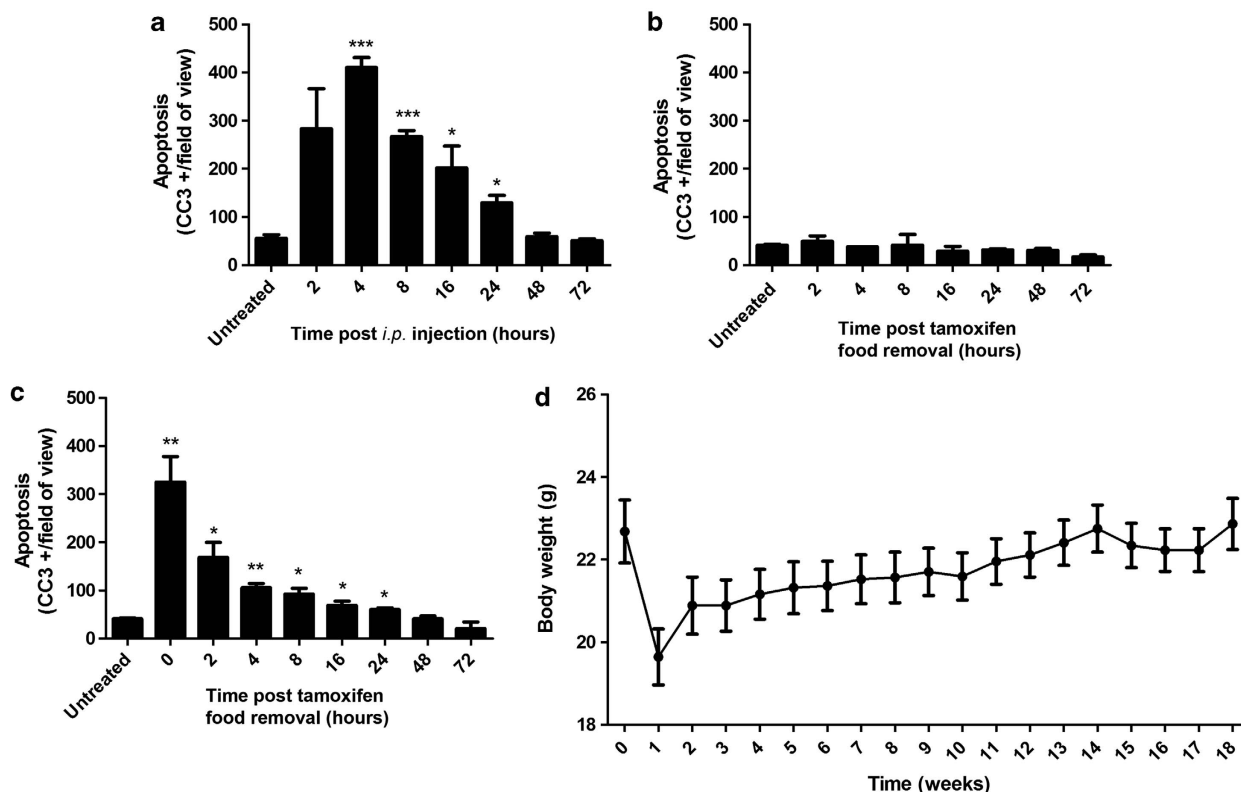


Figure 2. Restoration and persistence of p53 functionality in $p53^{K1/K1}$ mice after different routes of tamoxifen administration, as assessed by p53-dependent DNA damage responses in radiosensitive tissues. Tamoxifen was administered to $p53^{K1/K1}$ mice by one of three methods: (a) 1 mg i.p. injection of tamoxifen in oil; (b) Exposure to tamoxifen diet for 24 h; (c) Exposure to tamoxifen diet for 7 days. Following tamoxifen exposure, mice were irradiated with 2.5 Gy at the indicated time points post tamoxifen removal and tissues collected 4 h later. Any p53-dependent radiological response was assayed by quantitating apoptosis in thymus using cleaved caspase-3 (CC3) as an immunohistochemical marker. Mean and s.e.m. shown, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, compared with untreated samples, calculated by *t*-test, $n \geq 3$. (d) Body weight of mice supplied with tamoxifen-containing diet was assessed weekly (mean and s.e.m. shown; $n = 44$). Immunohistochemistry was performed on paraffin-embedded 4 μm sections which were deparaffinised, treated with citrate buffer for epitope retrieval and endogenous peroxidases blocked in 3% H_2O_2 . Staining was performed using the Rabbit VECTASTAIN ELITE ABC horseradish peroxidase kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. Primary antibody: anti-caspase-3 antibody (Cell Signalling Technology, Danvers, MA, USA; clone: 5A1E 1:50). Quantification was carried out by counting positive stained cells per field of vision, and two representative fields per mouse per time point were chosen for counting ($n \geq 3$ mice). For i.p. injections, tamoxifen base (T5648, Sigma-Aldrich, St Louis, MO, USA) was dissolved at 100 mg/ml in 100% ethanol by heating to 55 $^\circ\text{C}$ for 10 min, diluted in autoclaved sunflower oil to give a final concentration of 10 mg/ml and stored at -20 $^\circ\text{C}$ until use. Before i.p. injection, tamoxifen in oil was warmed to 55 $^\circ\text{C}$ for 10 min. Dietary tamoxifen was delivered in Tam400/CreER tamoxifen pellets (400 mg tamoxifen citrate per kg supplemented standard diet Global 2016 pellets), supplied by Harlan Teklad Europe, Boxmeer, The Netherlands. All procedures with animals were carried out in accordance with Home Office UK guidelines.

all tissues irrespective of tamoxifen provision (Supplementary Figure 3B).

To examine directly the rate of metabolism/excretion of tamoxifen in each experimental system, we determined the level of 4-OHT, the ER^{TAM}-activating tamoxifen metabolite, in the blood plasma of pure *C57BL6/J* mice (Supplementary Figure 4) using LC/MS (liquid chromatography–mass spectrometry). These studies confirmed that plasma 4-OHT is evident 2 h post single i.p. administration of 0.8 mg tamoxifen, returning to background levels after 24–48 h. Sustained dietary administration of tamoxifen-containing food for 7 days resulted in significant plasma 4-OHT and this fell to undetectable levels between 8 and 24 h after surcease of dietary tamoxifen. Thus 4-OHT, once tamoxifen has been ingested or injected, is cleared from tissues within 24 h, attesting to the need for repeated daily administration of tamoxifen in situations to maintain ER^{TAM} fusion protein function. Once animals have become acclimatized to dietary tamoxifen for at least 7 days they receive on average a 0.8 mg daily dose of tamoxifen over a 24-h period from grazing on food. This is reflected in the observation that 4-OHT levels and

corresponding functional readout of ER^{TAM} protein activity is lower than seen via a single 1 mg i.p. injection and decays to background levels rather more rapidly. Nonetheless, we guess that tamoxifen delivery via the food maintains more consistent ER fusion activity than can periodic i.p. delivery. Of note, in this study we used either a mixed *129-C57BL6/J* or pure *C57BL6/J* mouse strains—although we have not observed any strain-dependent differences in tamoxifen availability, this remains a possibility that should be considered in further *in vivo* studies using switchable ER fusion proteins.

To investigate the possibility of any adverse impact on general health through long-term administration of tamoxifen in food, mice were placed on a tamoxifenized diet for 18 weeks. Over the first week, mice lost an average of 16% body weight, apparently due to aphagia, but then recovered normal body weight and thereafter remained healthy, with no abnormalities detectable in any tissues (Figure 2d). Given the hazards associated with repeated i.p. injection, our data confirm the administration of tamoxifen in food as an effective and ethically preferable means of maintaining ER^{TAM} activity for prolonged periods *in vivo*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CHW, IG, AP and GIE designed the experiments; CHW, AP, IG and JA performed the analysis; CHW, IG, TDL and GIE wrote the article.

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