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ADMINISTRATION OF ANTENATAL GLUCOCORTICOIDS AND POSTNATAL SURFACTANT AMELIORATES RESPIRATORY DISTRESS SYNDROME-ASSOCIATED NEONATAL LETHALITY IN *ERK3*$^{-/-}$ PUPS

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Abstract

BACKGROUND—Respiratory distress syndrome (RDS) persists as a prevalent cause of infant morbidity and mortality. We have previously demonstrated that deletion of *Erk3* results in pulmonary immaturity and neonatal lethality. Using RNA-Seq, we identified corticotrophin releasing hormone (CRH) and surfactant protein B (SFTPB) as potential molecular mediators of *Erk3*-dependent lung maturation. In this study, we characterized the impact of antenatal glucocorticoids and postnatal surfactant on neonatal survival of *Erk3* null mice.

METHODS—In a double crossover design, we administered dexamethasone (dex) or saline to pregnant dams during the saccular stage of lung development, followed by postnatal surfactant or saline via inhalation intubation. Survival was recorded, detailed lung histological analysis and staining for CRH and SFTPB protein expression was performed.

RESULTS—Without treatment, *Erk3* null pups die within 6 hours of birth with reduced aerated space, impaired thinning of the alveolar septa, and abundant PAS-positive glycogen stores; as described in human RDS. The administration of dex and surfactant improved RDS-associated

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lethality of *Erk3*$^{-/-}$ pups, and partially restored functional fetal lung maturation by accelerating the down-regulation of pulmonary CRH and partially rescuing production of SFTPB.

CONCLUSION—These findings emphasize that Erk3 is integral to terminal differentiation of type II cells, SFTPB production, and fetal pulmonary maturity.

Introduction

Erk3 is a member of the MAP kinase family of serine/threonine kinases that play a key role in transducing environmental stimuli into a wide range of intracellular responses (1,2). The expression of *Erk3* is temporally regulated during embryonic development, increasing at the time of early organogenesis and declining after birth (3–5). The highest expression of *Erk3* is found in specific regions of the brain, lungs, skeletal muscle and gastrointestinal tract (3,6,7). In the lung, *Erk3* is restricted to the distal lung epithelium during the pseudoglandular phase, but shifted to the proximal airways during the saccular stage (8).

Targeted disruption of the *Erk3* gene in mice results in intrauterine growth restriction (IUGR) and lung immaturity with subsequent lethality (7). Histologic and morphogenic characterization indicate that the type II pneumocytes of *Erk3*$^{-/-}$ mice differentiate to the stage of being able to synthesize surfactant associated proteins, but cannot complete full functional development (as demonstrated by abundant intracellular glycogen with early neonatal lethality from RDS) (7). We have recently extended these studies at the whole transcriptome level. RNA sequencing (RNA-Seq) analysis of the lungs of wild type and *Erk3*$^{-/-}$ mice revealed differential expression of genes related to glucocorticoid induced lung maturation pathway. Specifically, we have observed down regulation of corticotrophin releasing hormone (*Crh*) in dexamethasone treated lungs compared to saline. This is temporally associated with up regulation of surfactant B (SFTB) production, which is *Erk3* dependent.

The purpose of the current study was to better define the role of *Erk3* in lung maturation and in regulation of CRH and SFTPB expression, and to investigate the potential role of antenatal glucocorticoid therapy and exogenous surfactant in preventing severe respiratory distress and early death in newborn *Erk3* null pups.

Methods

Animal husbandry and survival analysis

Animals were housed under pathogen-free conditions according to the procedures and protocols approved by the IRB at Baylor College of Medicine. For breeding, heterozygous males and females were intercrossed in mixed C57BL/6 × 129/Sv background. The presence of a vaginal plug indicated the beginning of gestation (E0.5).

Pups were attended at the time of delivery, and weighed at birth; total litter counts and pup weights were recorded and pups were marked. In a double crossover design, we administered subcutaneously 0.4 mg/kg prenatal dexamethasone (Sandoz Inc., Princeton, NJ) or saline at E17.5 and E18.5, alongside phospholipids-enriched surfactant (Survanta (beractant) 25 mg/ml suspension, Abbott Nutrition, Columbus, OH) or saline via inhalation

intubation at birth. Confirmation of pulmonary Survanta receipt was visualized by confirming indigo carmine co-stain at the level of the lungs, and neonatal survival with successful feeding was confirmed by the presence or absence of a milk spot.

There were 3 experimental groups; each group contained 16 littermates with an average of 6 to 8 pups per litter. Group 1 received one dose of surfactant; lungs, brain, heart and liver were harvested and phenotype analysis was performed at 24 hours; survival was calculated as the number alive at 24 hours. Group 2 received one dose of surfactant, and organ harvest and phenotype were done at 72 hours; survival was calculated as the number alive at 72 hours. Group 3 received three doses of surfactant every 12 hours and organ harvest and phenotype were done at 7 days. Survival was recorded every 2 hours for the first 6 hours, every 6 hours up to 48 hours, every 12 hours until 72 hours and every 24 hours until 7 days (Figure 1).

Administration of surfactant

Inhalation intubation technique: Pups where placed 80% supine and braced at the level of the scapula. A soft blunt end micropipette tip was introduced into their open mouths, and placed as proximal to the trachea as possible. Either saline or surfactant at 50 mg/kg of the phospholipid component was given. Survanta contains 25 mg of the phospholipid component per 1 ml, so the volume averaged between 2 and 3 microliters dependent upon pup weights and was rapidly administered over 30 seconds. For the administration, the entire litter was removed briefly from the cage at once, and returned after all pups received the solution. Confirmation of pulmonary Survanta was visualized by mixing surfactant with indigo carmine 0.08% (Akron Inc.) in a 10: 1 dilution to enable identification of surfactant receipt as a grossly visible blue stain at the level of the thorax or subscapular, through the thin neonatal skin.

Detailed lung histology

Lungs were harvested at detailed time intervals, then fixed and sectioned via systematic uniform random sampling (9–11). Each slide was examined for quantitative morphometric of PAS (glycogen laden) staining (12), airspace quantification (dissector and section area) (9,13) and septal thickness (Image ProPlus) (9,12).

Immunohistochemistry

Protein Immunohistochemistry (IHC) was performed on lung sections harvested at indicated time intervals post natal. Primary antibodies employed were CRH rabbit polyclonal (Abcam) and SFTPB rabbit polyclonal (Millipore). Unstained paraffin sections were deparaffinized in four changes of xylene for five minutes each and rehydrated through a series of graded alcohols with a final rinse in distilled water. Endogenous peroxidase was quenched by soaking sections in two solutions of 3% H_2O_2 in methanol for 10 min at room temperature. Prior to staining, antigen retrieval was performed to facilitate antibody binding to antigen. Slides were incubated at 99°C under pressure for 30 min in either Tris-EDTA buffer pH 8.0 (Sigma-Aldrich, St Louis, MO). No antigen retrieval step was required for staining with anti-SFTPB. Slides were then allowed to cool down for 10 min in the same solution, rinsed in three changes of distilled water, and placed in Tris Buffered Saline with

Tween 20 pH 7.4 (Signet Pathology Systems, Inc., Dedham, MA) for five min to decrease surface tension and facilitate coating by the subsequent reagents. The PolyVue HRP/DAB non-biotin polymer detection system (Diagnostic Biosystems, Pleasanton, CA) was used in the immunostaining protocols for CRH, while the Rabbit-on-rodent Detection System (Biocare Medical, Concord, CA) was used for SFTPB. Incubations occurred at room temperature unless otherwise specified, and for each step the sections were coated with 200 micro-liters of reagent. Tris buffered saline with Tween 20 pH 7.4 was used to rinse the sections between each of the immunohistochemistry steps. Background Sniper solution (Biocare Medical, Concord, CA) was used to block non-specific staining for 10 min at room temperature. The Primary antibody was diluted using Renaissance antibody diluents (Biocare Medical, Concord, CA), at a dilution of 1:400 for CRH, and 1:2000 for SFTPB. Slides were incubated with the primary antibody solution overnight at 4°C. Sections were then incubated in the universal secondary antibody provided with the kit for 15 min, followed by the HRP label reagent. Afterwards, Stable DAB Plus (Diagnostic Biosystems, Pleasanton, CA) was applied for 5 minutes as chromagen. The slides were rinsed in distilled water and manually counterstained with Harris Hematoxylin (Fisher Scientific,) for 15–30 seconds and rinsed in distilled water. Coverslips were then applied to each slide using synthetic glass and permount mounting media. Negative controls and non-specific antibodies were included in each immunostaining procedure and both were negative. *SFTPB:* Immunostained slides were examined by reviewers masked to whether the tissue originated from an animal exposed to corticosteroid treatment or not. For each slide examined, five random high-power fields were graded using a 0 to 3 scale where 0 indicated the absence of positive staining, and 3 indicated intense and diffuse positive staining. *CRH:* Immunostained slides were examined by reviewers masked to whether the tissue originated from an animal exposed to corticosteroid treatment or not. For each slide examined, five random high-power fields were graded using a 0 to 5 scale where 0 indicated the absence of positive staining, and 5 indicated intense and diffuse positive staining. For both the location of positive staining areas was also recorded.

Statistical analysis

Survival analysis: The data was analyzed with Kaplan-Meier survival analysis using Sigma Plot 11 with minimal significance designated at $p<0.05$. *Lung histology analysis*: Data was analyzed with ANOVA or an independent samples t-test as appropriate using SPSS V 11.5 with minimal significance designated at $p<0.05$, N=5. *IHC analysis*: The average of all grades was calculated for each slide, and IHC grades of were compared across the treated and untreated groups using the independent sample T-test after the equal variance test was performed using the statistical software package SPSS v 11.5 with minimal significance designated at $p<0.05$, N=5.

Results

Demonstrated impact of antenatal dexamethasone and postnatal surfactant on neonatal survival

Three groups of mice were analyzed in this double crossover study (Figure 1). Group 1 received one dose of surfactant or saline and was followed for a period of 24 h (neonatal

demise or sacrifice for histology at 24 h); group 2 similarly received one dose of surfactant or saline but was followed for up to 72 h; and group 3 received three doses of surfactant or saline and was followed up to one week of life. Group 1 showed a mean survival increase from 6 ±0 h to 16.2 ±2.4 h among mice receiving dex/surfactant versus saline/saline, with 57% surviving to 24 h (Figure 2A). When the observation period was extended to 72 hours, there was a significant difference in mean survival with lengthening from 4.3 ±0.7 h to 28.1 ±4.4 h ($p=0.01$), with 38% of pups surviving to 72 h (Figure 2B). We therefore reasoned that the initial benefit of surfactant may be lost after 24 hours, and next tested whether repeat dosing would improve the interval to survival. The mean survival improved from 1.5 ±0.5 h to 59.5 ±21.6 h ($p<0.01$), with 35% of animals surviving to 1 week without evidence of RDS in this group (Figure 2C). In all 3 groups, survival of both *Erk3*+/+ (Figure 2D) and +/− (Figure 2E) littermates were not altered by drug or control treatment, and there was no significant difference among homozygous and heterozygote littermates.

Detailed histological analysis of the lungs

To assess the cellular impact of glucocorticoid and surfactant therapy, dams were treated with dexamethasone or saline at E17.5 and E18.5 of gestation, and *Erk3*$^{-/-}$ pups (identified by rapid genotyping) received either surfactant or saline at birth. Lungs were harvested at 3 h of life and processed for histological analysis. Following strict histology guidelines (9), we observed that *Erk3*$^{-/-}$ mice receiving antenatal dexamethasone and postnatal surfactant administration demonstrate increased aerated lung area when compared to saline or dexamethasone alone ($p\leq0.05$; Figure 3B) with thinner septal membranes. ($p<0.01$; Figure 3C). Dexamethasone alone and the combination of dexamethasone and surfactant were equally efficacious in reducing functionally immature glycogen laden pneumocytes ($p<0.01$), but dexamethasone alone did not significantly alter neonatal survival relative to combination therapy although on group 1 and 2 (Figures. 2A and 2B, dex alone with an observed 30% effect on neonatal survival) probably due to a hydration effect by the administration of saline at birth. These results suggest that *Erk3* null pups are dying in early neonatal period secondary to a condition histologically and functionally analogous to RDS in humans.

Immunohistochemistry analysis

Using a double crossover design, we administered antenatal dexamethasone or saline at E17.5 or E18.5. At birth, newborn pups of *Erk3*$^{-/-}$ and *Erk3*$^{+/+}$ genotypes received either surfactant or saline, and lung sections were obtained at 3 or 6 hours of life. By IHC analysis, *Erk3*$^{-/-}$ pups treated with antenatal dexamethasone and postnatal surfactant demonstrated persistent attenuation of CRH expression (Figure 4A) when compared with controls (saline/saline ($p<0.001$)) and relative to dexamethasone alone ($p<0.05$; Figure 4C). *Erk3*$^{+/+}$ pups also showed attenuation of CRH expression when dex was given ($p<0.05$; Figure 4D) and when dex/surfactant were used ($p<0.05$; Figure 4D)

Moreover, administration of antenatal dexamethasone and postnatal surfactant in *Erk3*$^{-/-}$ pups persistently demonstrated attenuation of CRH up to 6 h of life when compared to controls (saline/saline, $p<0.01$; Figure 5B). This is also demonstrated in a qualitative analysis in the photomicrographs presented in Figure 5A.

Dexamethasone and surfactant treated *Erk3*$^{-/-}$ pups demonstrated increased SFTPB production relative to antenatal dexamethasone alone ($p<0.001$) or saline alone ($p=0.025$; Figure 4E). There is no deviation from saline/saline treated mice on *Erk3*$^{+/+}$ pups treated with dex or dex/surfactant. For Erk3$^{-/-}$ at 6 hours of life there is an increase in SFTPB relative to saline alone demonstrated in a qualitative analysis in the photomicrographs presented in Figure 5C. This further confirmed in a quantitative analysis ($p=0.006$; Figure 5D). Functional and histologic findings significantly correlated with SFTPB expression *in situ* (Figure 4B).

Discussion

We have developed a novel murine model with which to study the molecular mechanisms underlying functional pulmonary maturation and developmental of neonatal RDS. We have demonstrated that administration of antenatal glucocorticoids (dex) and postnatal surfactant significantly improve neonatal RDS and survival in an *Erk3* null murine model.

These studies have necessitated several technical innovations which are of likely interest to translational investigators, including neonatal drug inhalation. In a pilot study to determine the best method of surfactant administration, we initially tested three distinct techniques. The only published literature of drug instillation techniques in mice employs significantly larger juvenile or adult animals (14–16). Intubation and tracheostomy proved infeasible due to the newborn pups' small size. Drug administration itself could also be fatal. We initially attempted to modify the tracheal installation technique used in adult mice on our pups (17,18) using a syringe with an 26 gauge needle to inject surfactant through the neck directly into the trachea, but found that mortality was high with this technique. The use of nasal instillation, wherein surfactant or saline is administered through the nares, was similarly unsuccessful. Administration time was 60 min for half of the dose, and the pups became hypothermic and many died during the treatment. Finally, we modified the inhalation intubation technique (19,20). Using a micropipette, we placed the semiflexible blunted pipette tip as close to the trachea as possible through the pups' open mouths and administered the solution (saline or surfactant). We found that if the administered solution was delivered to the lungs, bubbles would arise from the nares. To further confirm pulmonary surfactant delivery, we stained the saline or surfactant solution with indigo carmine and were able to visualize the color at the level of the lungs through their skin. While we found that the inhalation intubation technique is reproducible, we cannot verify that all of the dosage reached the lungs. In published studies using this technique in adult mice, approximately 85% enters the lungs (19). These results are comparable to intratracheal injection (19,21).

We used Survanta, a pulmonary surfactant suspension from natural bovine lung extract containing phospholipids, neutral lipids, fatty acids and surfactant-associated proteins SFTPB and SFTPC. It does not contain SFTPA (22). We chose this solution because Survanta has the lowest concentration of surfactant protein B. Survanta contains 25 mg phospholipids per ml of solution (22,23). Current published studies reference surfactant dosages from 50 to 200 mg/kg in adult mice and there is no significant difference in dose response (17,24,25). We therefore selected the lowest recommended dose, 50 mg/kg of the

phospholipid component, in order to administer the lowest possible volume (26) as it is less traumatic to the pups. Similarly, we moved the administration of antenatal glucocorticoid from E16.5 and E17.5 used in previous studies (7) to E17.5 and E18.5 to more closely correspond with the saccular stage of lung development. This is the same stage when glucocorticoids are used in human development to improve lung maturity for preterm infants (27–29).

We have previously demonstrated uniform neonatal lethality due to lung immaturity by 6 hours of age among *Erk3*$^{-/-}$ pups [7]. With the introduction of surfactant among all groups of Erk3 null pups, neonatal surfactant with antenatal dexamethasone resulted in enhanced survival. Our observation of partial phenotypic abrogation with antenatal glucocorticoid administration and postnatal surfactant administration provides strong evidence for an *Erk3* pathway of type II pneumocyte differentiation and maturation.

In our previous publication, histological analysis of *Erk3*-deficient lungs revealed that overall organogenesis of the lungs was preserved and there were no obvious defects in branching morphogenesis (7). We therefore sought to now assess the differentiation of type II pneumocytes by analyzing the expression of pulmonary surfactant associated protein C (SFTPC) and measuring the content of cytoplasmic glycogen, which serves as a substrate for surfactant phospholipids (30), in lungs from E18.5 embryos. Immunoreactive SFTPC was not altered in *Erk3*$^{-/-}$ mice. This is in accordance with our prior ultra-structural analysis by electron microscopy revealing that type II pneumocytes from *Erk3 null* mutant mice contain abundant glycogen granules (qualitative and quantitative) as compared to controls. Antenatal glucocorticoids partially rescued type II pneumocyte differentiation restoring glycogen levels to those of normal WT mice. Nevertheless, they had no effect on neonatal morbidity nor did they rescue *Erk3*-mediated modification of fetal growth potential.

In this publication we have further characterized lung histology of *Erk3* null mice demonstrating that their lethality is related to the classic histology described in RDS in humans. The replacement of surfactant in this mouse model, restored the glycogen content of type II pneumocytes to normal levels, and improved their architecture. The discrepancy between the data presented herein (showing no difference between dexamethasone alone and saline controls in terms of airspace area) compared to our prior published data (demonstrating distinctions in the airspace area between dexamethasone and saline in WT lungs) likely relates to the time of collection of the neonatal lungs. Specifically, in our prior study, pups were delivered by cesarean at E18.5 and sacrificed immediately in order to study the *in utero* variation. In the current study, our aim was to allow for surfactant administration and thus pups were delivered vaginally and tissue was collected at the time of scheduled postnatal/neonatal sacrifice (*i.e.*, 3 hours of age and greater). Thus the pups insulflated their lungs, resulting in the equivalency shown herein. Although the surfactant effect on survival nadirs after 24 hours, taken together these findings suggest that *Erk3* is integral to terminal differentiation of type II cells, SFTPB production, and fetal pulmonary maturity in the immediate perinatal interval.

The association of the attenuation of pulmonary CRH by administration of glucocorticoids is consistent with data from an unrelated mouse model of CRH deficiency (31) suggesting a

fetal glucocorticoid requirement for lung maturation (32). Postnatally, the CRH KO mice demonstrated normal growth and longevity, suggesting that the major role of glucocorticoid is during fetal rather than postnatal life. The attenuation of pulmonary CRH in our *Erk3* null mice combined with published data in humans showing high levels of CRH in maternal serum correlating with fetal lung maturity suggests the existence of a feedback loop between CRH and fetal lung maturation. Moreover it indicates that CRH is a significant molecular mediator of pulmonary maturation.

CRH has been put forward as a key neuroendocrine modulator of pregnancy and parturition (33–37). During mid-gestation there is a rapid increase in the concentration of CRH in the maternal plasma (38). This occurs alongside production of surfactant in the fetal human lung beginning after week 24 of gestation, which corresponds to the saccular stage of lung development and the earliest consistently documented threshold of viability. The second peak in CRH concentration occurs during the last few weeks of pregnancy (33). In groups matched by gestational age in proximity to term, larger mean values of CRH are observed among neonates in which the lecithin/sphingomyelin ratio was greater than 2 or the phosphatidylglycerol test for lung maturity was positive (39). These could explain a possible relationship between the peaks in CRH in human maternal plasma with the attenuation of CRH in the lungs that we find in our murine model. Further work is needed to discover what cells are producing or expressing CRH at the level of the lung and to continue to discover other molecular mediators of glucocorticoid-induced lung maturation.

In sum, we have demonstrated that neonatal surfactant combined with antenatal glucocorticoids enhances neonatal RDS-free survival in a murine model of *Erk3*-dependent pulmonary maturation and neonatal survival. Therefore, a likely explanation (but not mechanistically demonstrated *per se*) is that the treatment with antenatal dexamethasone partially restored functional fetal lung maturation by accelerating the down regulation of pulmonary CRH (*Erk3* indirect or independent effect because we also demonstrated attenuation in *Erk3* WT) and administration of postnatal surfactant partially rescued endogenous production of SFTPB (*Erk3* dependent). Collectively, our results suggest a functional role to the elusive atypical MAP kinase Erk3, demonstrating its essential function in terminal functional differentiation of type II pneumocytes and SFTPB production (Figure 6).

To date, no treatment completely prevents nor ameliorates RDS, and therapy is both costly and of limited benefit. Thus, there is a need to explore additional pathways to facilitate new therapeutic developments in this arena. Ongoing work to further elucidate the relationship of Erk3 with the development of RDS and SFTPB is one pathway which may lead to these developments. It is also unclear to what extent regulatory networks involved in type II cell maturation are involved in alveolar repair and reconstitution of surfactant homeostasis (*i.e.* bronchopulmonary dysplasia). Our described work herein will lay the foundation for these and future studies.

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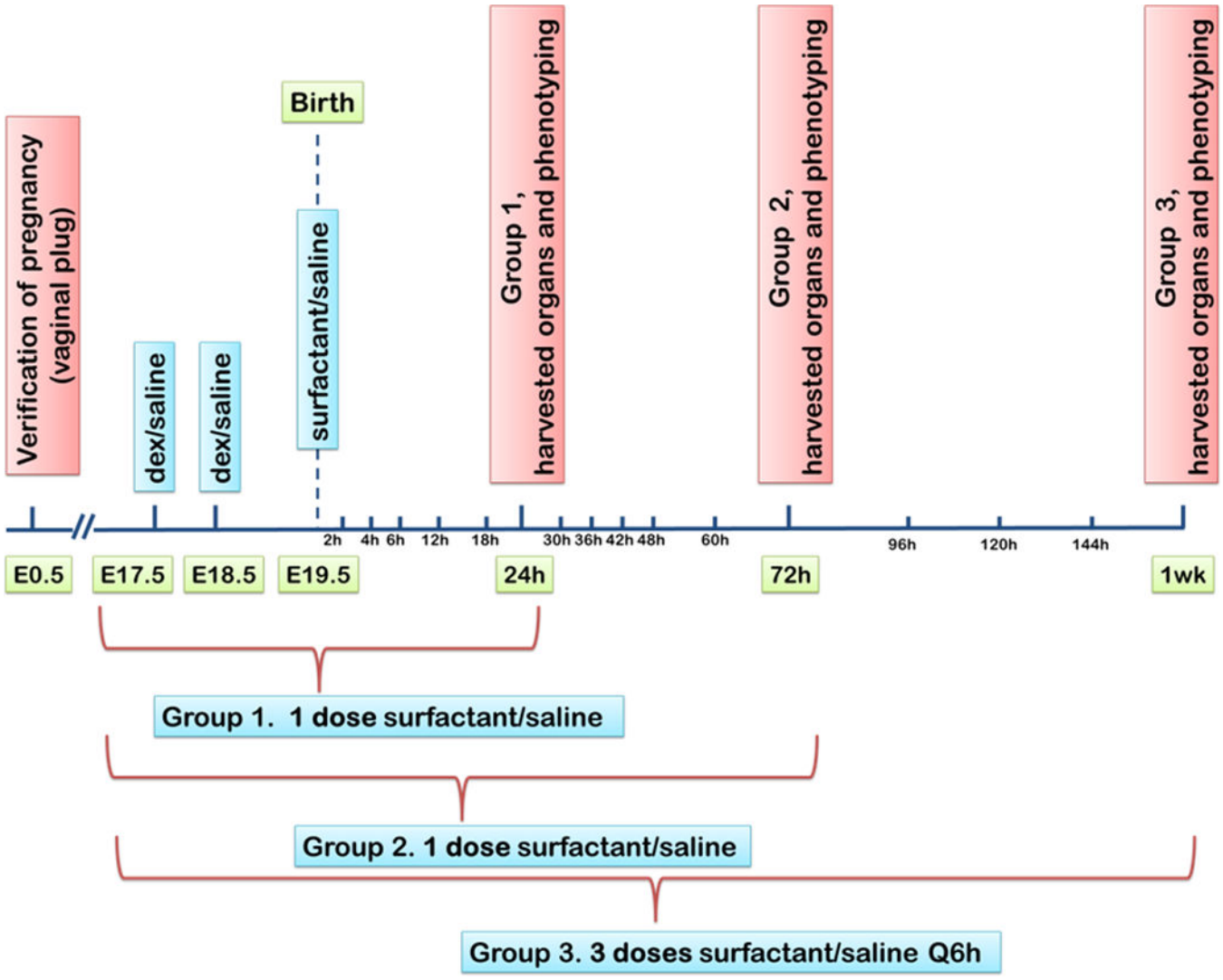

Figure 1. Timeline schematic of the double crossover design showing important events and defining the 3 groups

In the time line the green squares demark the time point for interventions. With the verification of pregnancy indicated by the presence of vaginal plug on E0.5, timing of antenatal administration of dex/saline as thereafter designated. Birth uniformly occurred on E19–19.5, and deliveries were attended in order for the pups to be weighed, marked, and precise timing for rate of live birth survival to be calculated. The initial dose of surfactant or saline control was administered at the time of birth. Ongoing direct investigator observation and identification of neonatal demise versus survival occurred at the indicated time points (black lettering). As noted, this occurred every 2 hours for the first 6 hours of life, then at 6 hour intervals through 48 hours. The light blue squares list each group definition. In all groups dams received dex or saline then newborn pups of group 1 and 2 received 1 dose of surfactant, but in group 1 organs were harvested at 24 h and in group 2 organs were harvested at 72 h. In contrast group 3 received 3 doses of surfactant/saline every 12 h and organs were harvested at 1 week. Rate of survival was calculated at 24 hours (Group 1), 72

hours (Group 2) and 7 days (Group 3). Genotyping occurred at the time of neonatal sacrifice, thus investigators were blinded to genotyping during the course of the experiment.

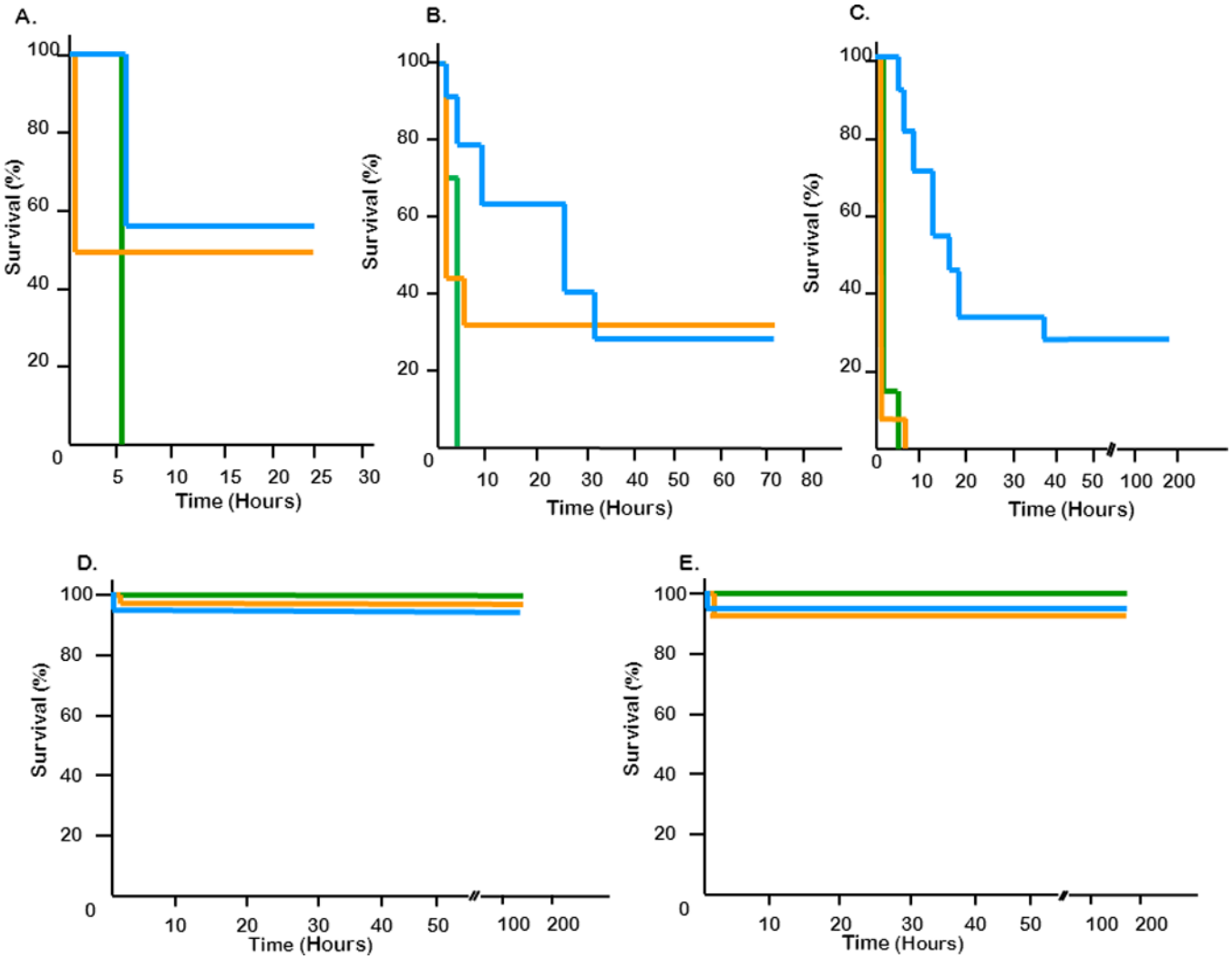

Figure 2. Enhanced free-RDS survival of *Erk3* KO with dexamethasone/surfactant administration

Survival curves where Y axis represents survival in percentages and X axis represents time in hours, green represents saline/saline, orange is dex/saline and light blue is dex/surfactant. **(A)** *Group 1 (sacrificed at 24 hours, n=16): Erk3*−/− mice that received antenatal dex or saline and 1 dose of postnatal surfactant or saline were compared to saline/saline observed up to 24 h. Mean survival lengthened from 6±0 to 16.2±2.4 h with 57% surviving to 24 h. **(B)** *Group 2 (sacrificed at 72 hours, n=26): Erk3*−/− mice that received antenatal dex or saline and 1 dose of postnatal surfactant or saline were compared to saline/saline observed up to 72 h. Mean survival lengthened from 4.3±0.7 to 28.1±4.4 h (p=0.01) with 38% surviving to 72 h. **(C)** *Group 3 (sacrificed at 7 days, n=30): Erk3*−/− mice that received antenatal dex or saline and 1 dose of postnatal surfactant or saline were compared to saline/saline observed up to 7 days. Mean survival improved from 1.5±0.5 to 59.5±21.6 h (p<0.01) with 35% surviving to 1 week without evidence of RDS. **(D)** *Group 1,2 and 3 (n=30); Erk3*+/+ littermates were observed with <5% mortality observed on all groups. **(E)** *Group 1, 2 and 3 (n=30); Erk3*+/− heterozygote littermates. Less than 8% mortality observed for

all groups. There was no significant difference in survival among *Erk3*+/+ and +/− littermates under all treatment conditions.

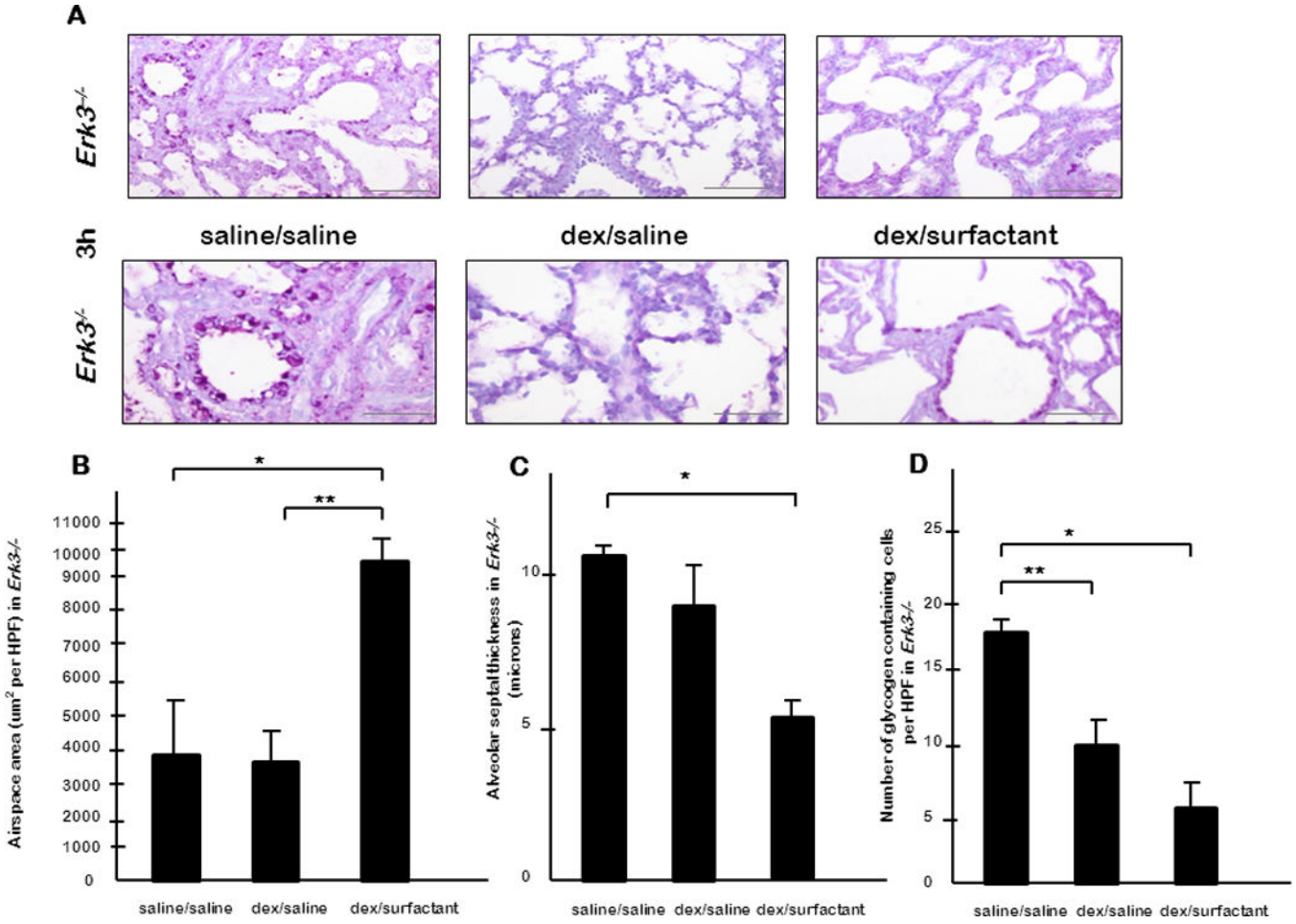

Figure 3. *Erk3*$^{-/-}$ pups demonstrate histologic and morphologic classical hallmarks of respiratory distress syndrome (RDS) at 3 hours of life

(A) Pregnant females from *Erk3* intercrosses were treated with dexamethasone or saline as described. In order to avoid potential artifact from end stage RDS, neonates were sacrificed at 3 hours postnatal. Lung sections were stained with PAS staining. Magnification: top row 20× and bottom row 40×. Histologic sections demonstrative of 5 littermates in each treatment group. **(*B*)** Airspace quantification (dissector and section area) in lung sections from *Erk3*$^{-/-}$ (n=5) as described for panel A. Antenatal dex combined with postnatal surfactant administration in *Erk3*$^{-/-}$ mice increased aerated lung area when compared to saline alone, or in combination with dex alone (**panel B**; *p=0.05, **p=0.03) alongside thinner septal membranes (**panel C**; p=0.01). Dex alone and the combination of dex and surfactant was equally efficacious in reducing histologic immature glycogen laden pneumocytes (**panel D**; **p=0.02, *p=0.01).

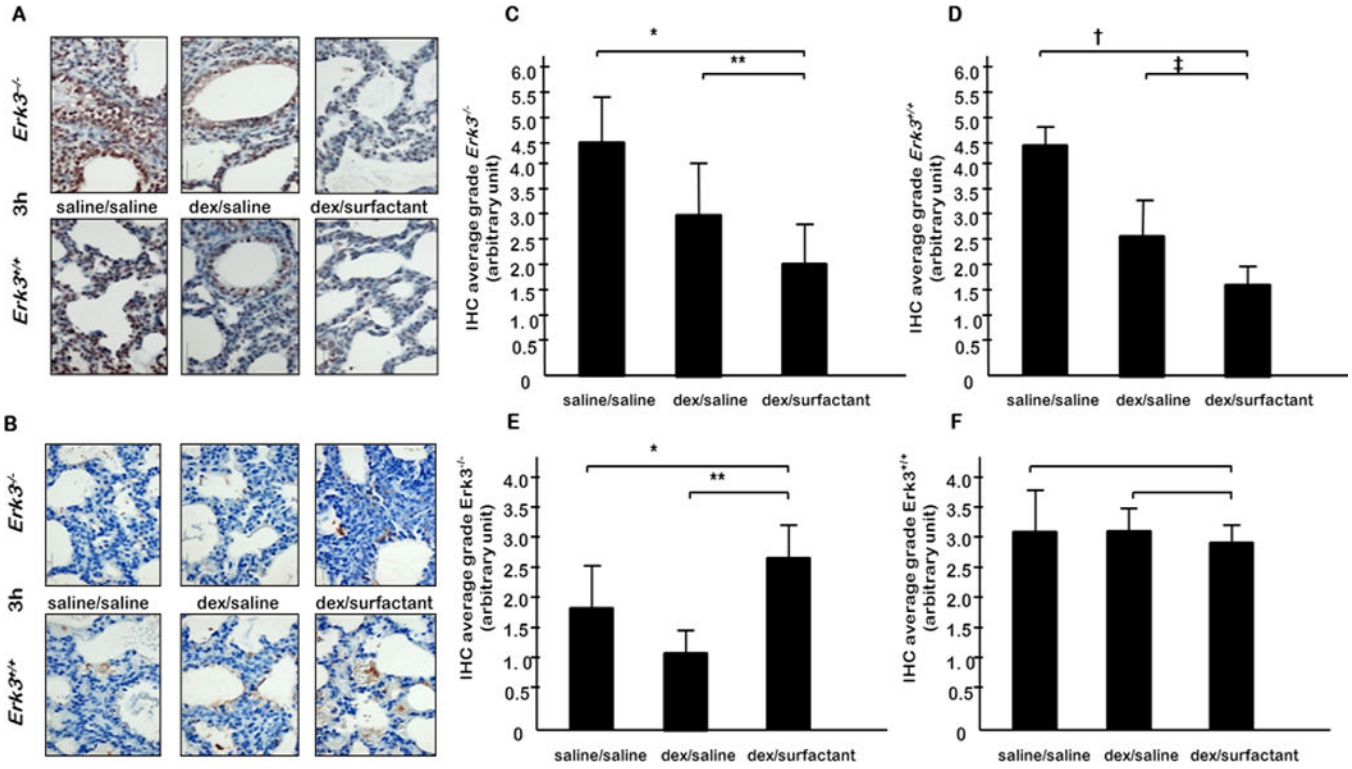

Figure 4. Antenatal dexamethasone accelerated CRH down regulation in both *Erk3*$^{+/+}$ and *Erk3*$^{-/-}$ and postnatal surfactant rescued SFTPB production among *Erk3*$^{-/-}$ pups at 3 hours of life

Dams from *Erk3* intercrosses were treated with dexamethasone or saline, and newborn pups were administered surfactant or saline. Lungs were harvested at 3 h of life. **(A)** *IHC of lung sections stained with DAB chromagen for Corticotrophin Releasing Hormone (CRH).* Magnification, 40×. **(B)** *IHC of lung sections stained with DAB chromagen for Surfactant Protein B (SFTPB).* Magnification, 40×. **(C)** *Quantitation of DAB chromagen staining in lung sections from Erk3*$^{-/-}$ *pups (n=5).* Significant attenuation of CRH with saline (*p<0.001) or dexamethasone alone (**p<0.05) compared to dexamethasone and surfactant. **(D)** *Quantitation of DAB chromagen staining in lung sections from Erk3*$^{+/+}$ *pups (n=5).* Significant attenuation of CRH with saline (†p<0.05) or dexamethasone alone (‡p<0.05) compared to dexamethasone and surfactant. **(E)** *Quantitation of DAB chromagen staining in lung sections from Erk3*$^{-/-}$ *pups (n=5).* Significant rescue of postnatal SFTPB production relative to antenatal dex (**p<0.001) or saline (*p=0.025). **(F)** *Quantitation of DAB chromagen staining in lung sections from Erk3*$^{+/+}$ *pups (n=5).* No significant difference in SFTPB expression.

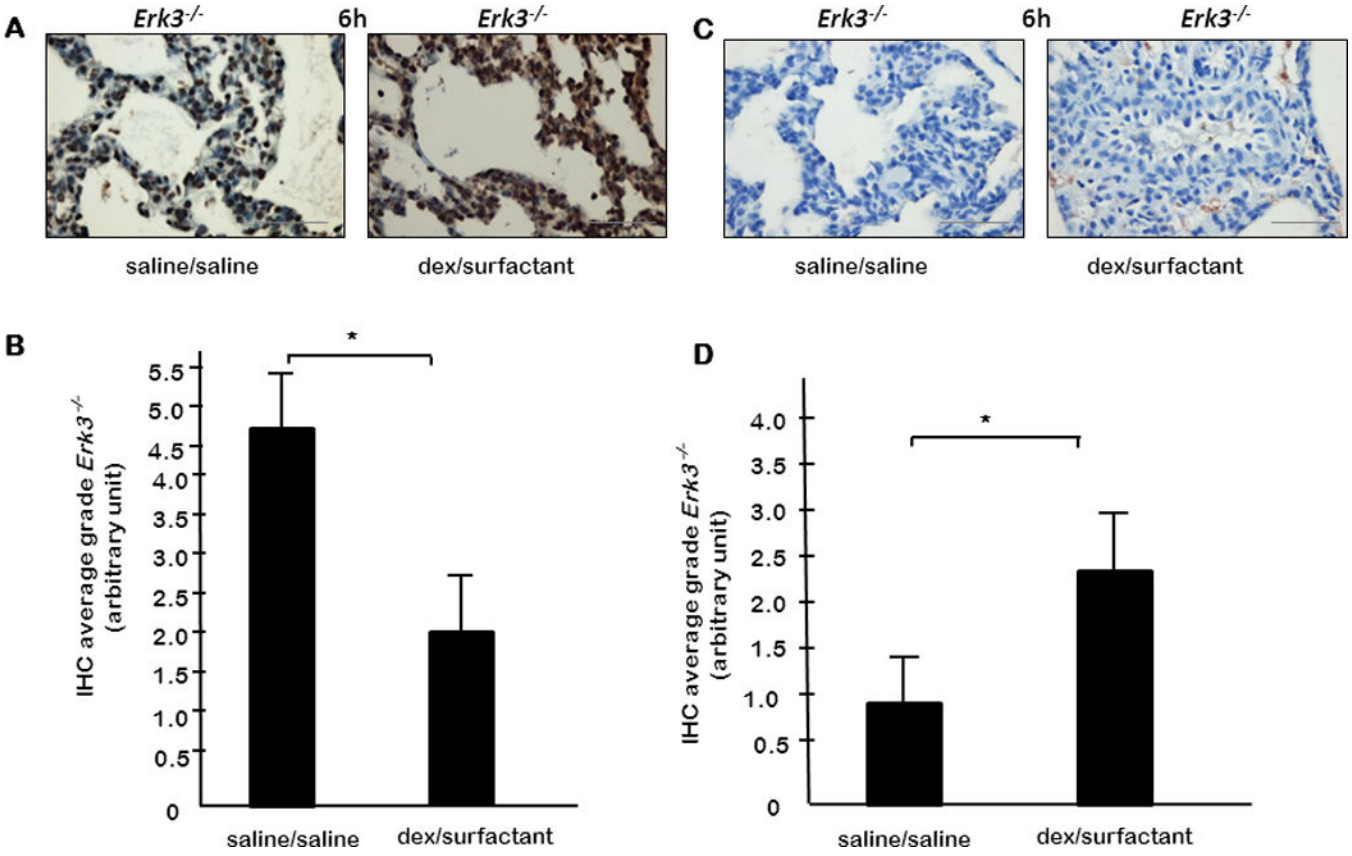

Figure 5. Antenatal dexamethasone and postnatal surfactant continue to down regulate CRH in Erk3 KO and postnatal surfactant rescued SFTPB production at 6 hours of life

Pregnant females from Erk3 intercrosses were treated with dexamethasone or saline alongside newborn pups who postnatally received surfactant or saline. Lungs were harvested at 6 hours of life. **(A)** *Immunohistochemistry photomicrographs of lung sections were stained with DAB chromagen for CRH.* Magnification, 40×. **(B)** *Quantitation of DAB chromagen staining in lung sections from Erk3*$^{-/-}$ *pups (n 5).* Significant attenuation of CRH is observed (*p<0.001). **(C)** *Immunohistochemistry photomicrographs of lung sections were stained with DAB chromagen for SFTPB.* Magnification, 40×. **(D)** *Quantitation of DAB chromagen staining in lung sections from Erk3*$^{-/-}$ *pups (n=5).* Significant increased SFTPB production relative to saline (*p=0.006) at 6 hours of life.

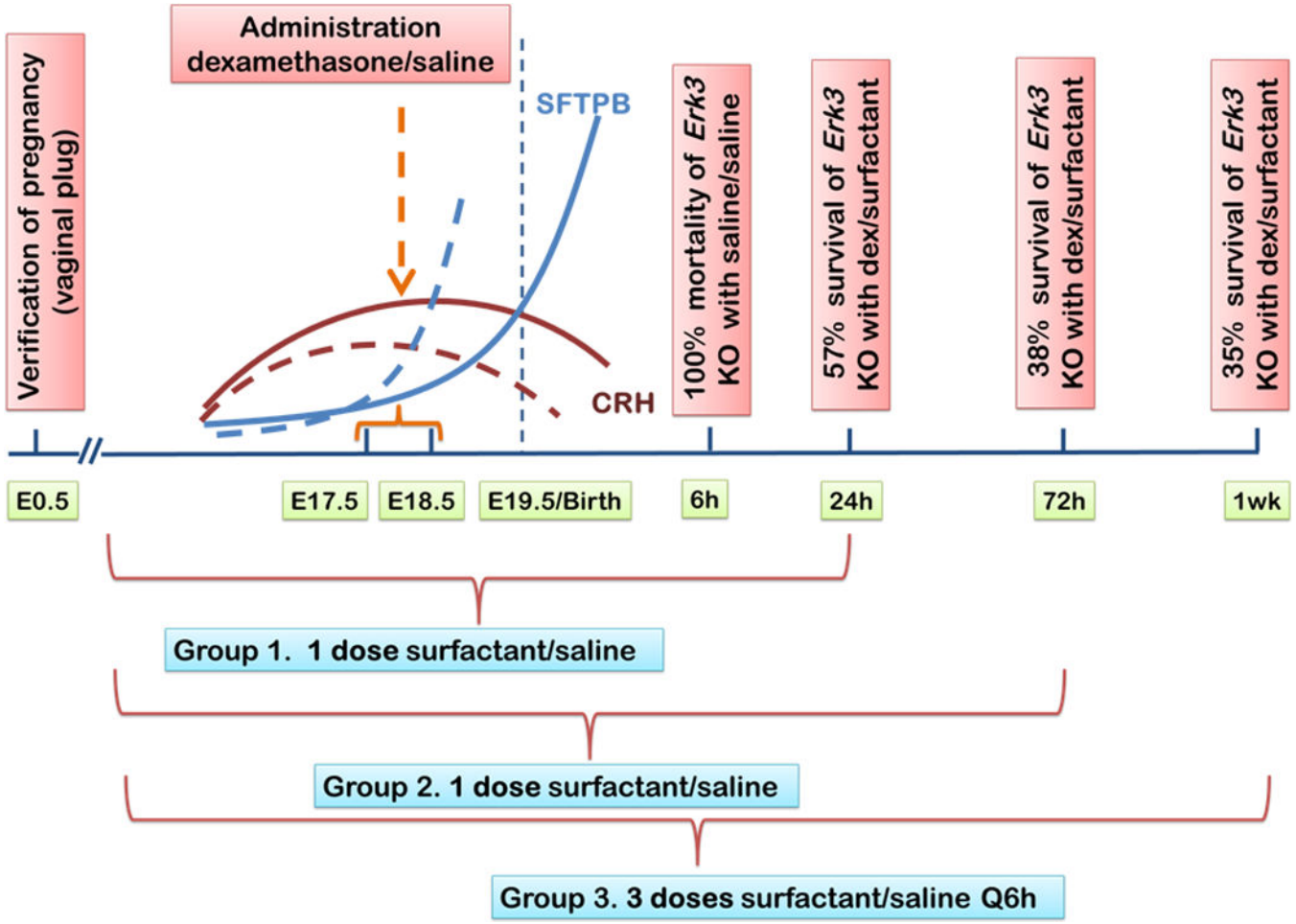

Figure 6. Administration of antenatal glucocorticoids (dex) and postnatal surfactant significantly improve neonatal RDS- free survival in an *Erk3* null murine model

Red and blue solid lines represent endogenous pulmonary CRH and SFTPB protein expression in lungs of *Erk3* null newborn pups that received control (saline) treatments. Dashed lines represent CRH and SFTPB levels when antenatal dexamethasone and postnatal surfactant is administered, which in turn leads to significantly improved neonatal survival as explained on the light pink squares.